Iron and Tuberculosis (TB) pathogenesis

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DECLARATION

By submitting this thesis, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

SUMMARY

Iron is an essential element that plays a role in the process of respiration, oxygen transport and as a principle cofactor to several enzymes. Iron homeostasis is a finely regulated process since excess levels become toxic to healthy cells *via* the production of reactive oxygen species. A plethora of genes that control several key points throughout this regulatory process have been identified. Research focusing on changes in expression levels and downstream functional effects of these genes has become increasingly important over the past decade. One area of particular interest has emerged since a link between iron status and host response to Mycobacterium tuberculosis infection was discovered. Although the prevalence of Tuberculosis has decreased across the globe with the exception of Africa and parts of Europe, the mortality rate remains high. Therefore, research that focuses on understanding an individual's predetermined susceptibility to TB infection at the genetic level could provide health care practitioners with the tools required to identify and educate at-risk individuals prior to TB infection.

RT-qPCR was utilised to determine expression profiles for eight iron genes (*CP, CYBRD1, FTH, FTL, LTF, HFE, HMOX1,* and *SCL40A1*) normalised to three reference genes (*ACTB, GUSB,* and *RPL37A1*). Up-regulation is demonstrated in the TB group for transcript levels recorded for *CYBRD1, HFE, HMOX1,* and *SLC40A1*. Several measured serum parameters including conjugated, unconjugated, total bilirubin, and total protein were increased in the TB group while albumin was significantly lower in this group. Correlation analysis demonstrated that a positive correlation exists between transferrin saturation and iron and a negative correlation exists between transferrin and ferritin levels. Individuals categorised with low serum iron levels demonstrated lower *CP*/*GUSB* levels and higher *HMOX1*/*GUSB* levels. Individuals categorised with low transferrin saturation levels demonstrated higher *FTL*/*GUSB* and *SLC40A1*/*GUSB* levels and lower *CP*/*GUSB.*

Results from this study provide further evidence for the relationship between iron status and TB infection rates, although protein studies are required to confirm these results. The data obtained illustrate the important role that these profiles and iron parameters may play in the clinical field when identifying at-risk individuals. Further investigation that focuses on which gene profile and parameter combinations show the most distinctive utility in the clinical setting is warranted.

OPSOMMING

Yster is ‘n noodsaaklike element wat ‘n rol speel in die proses van respirasie en die vervoer van suurstof en ook ‘n belangrike ko-faktor vir verskeie ensieme is. Yster homeostase is op ‘n fyn manier gereguleer omdat oormatige vlakke toksies kan wees vir gesonde selle wanneer reaktiewe suurstofspesies geproduseer word. ‘n Magdom gene wat verskeie sleutelpunte in hierdie proses kontroleer is voorheen identifiseer. Navorsing wat fokus op die veranderinge in geenuitdrukkingsvlakke en die funksionele gevolge daarvan het oor die afgelope dekade toenemend belangrik geword. Een gebied van spesifieke belang het na vore gekom nadat ‘n verband tussen ystervlakke en die manier waarop die immuunstelsel reageer op *Mycobacterium tuberculosis* infeksie, ontdek is. Alhoewel die voorkoms van Tuberkulose wêreldwyd, behalwe in Afrika en sekere dele van Europa, afgeneem het, bly die sterftesyfer hoog. Daarom kan navorsing wat daarop fokus om ‘n individu se voorafbepaalde vatbaarheid vir TB-infeksie op die genetiese vlak te verstaan dalk aan gesondheidswerkers die regte instrumente verskaf om hoë-risiko individue te identifiseer en op te voed voordat hulle TB ontwikkel.

RT-qPKR is gebruik om die geenuitdrukkingsvlakke van agt ystergene, wat met drie verwysings-gene (*ACTB, GUSB,* en *RPL37A1*) genormaliseer is, te bepaal. ‘n Toename in die uitdrukkingsvlakke van *CYBRD1*, *HFE*, *HMOX1*, en *SLC40A1* is in die TB-groep waargeneem. Die bloedvlakke van verskeie parameters insluitend gekonjugeerde, ongekonjugeerde, totale bilirubin, en totale proteïen was hoër in die TB-groep, terwyl albuminvlakke laer was in hierdie groep. Korrelasie-analise het ‘n positiewe korrelasie tussen transferrin-versadiging en yster getoon, terwyl daar ‘n negatiewe korrelasie tussen transferrin- en ferritinvlakke gevind is. Individue met lae ystervlakke het laer *CP*/*GUSB*-vlakke en hoër *HMOX1*/*GUSB*-vlakke getoon. Individue met lae transferrin-versadiging het hoër *FTL*/*GUSB*- en *SLC40A1*/*GUSB*-vlakke en laer *CP*/*GUSB*-vlakke getoon.

Resultate uit hierdie studie verskaf verdere getuienis dat daar ‘n verwantskap tussen ystervlakke en TB-infeksiekoerse bestaan, alhoewel proteïenstudies nodig is om hierdie resultate te bevestig. Die data dui op die belangrike rol wat hierdie profiele en ystervlakke in die kliniese veld mag speel in die identifisering van hoë-risiko individue. Verdere ondersoek, gefokus op watter geenprofiel en parameterkombinasies die grootste nut in die kliniese omgewing bied, is geregverdig.

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LIST OF ABBREVIATIONS AND SYMBOLS

^{o}C	degrees Celsius
^{o}C/s	degrees Celsius per second
%	percentage
β	beta
β2m	beta-2 microglobulin
µl	microlitre
µl/ml	microliter per millilitres
3'	3-prime
5'	5-prime
A	adenosine
ACTB	beta-actin protein
ACTB	beta-actin gene
ALT	alanine aminotransferase
AST	aspartate transaminase
B	boron
BCG	Bacille Calmette-Guérin vaccine
bp	base-pair
BLAST	basic local alignment search tool
C (Cys)	cysteine
C	cytosine
C	carbon
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CO	carbon monoxide
CP	crossing point
CP	ceruloplasmin protein
CP	ceruloplasmin gene
Cq	quantification cycle
CR3	complement receptor 3
CRP	C-reactive protein

Cu	copper
CUL1	cullin-1
CXCL12	CXC ligand 12
CXCR4	CXC receptor 4
CYBRD1	cytochrome b reductase 1 gene
D (Asp)	aspartate
DCYTB	duodenal cytochrome b reductase 1
dH_2O	distilled water
Dmt1	mouse divalent metal transporter 1
DMT1	divalent metal transporter 1
DNA	deoxyribonucleic acid
DOTS	directed observed treatment strategy
EDTA	ethylebediaminetetraacetic acid
et al.	and others
ERE (s)	estrogen response element (s)
EtBr	ethidium bromide
EtOH	ethanol
F	forward primer
FBXL5	f-box and leucine-rich repeat protein 5
Fe	iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FPN	ferroportin protein
FPN	ferroportin gene
FTN	ferritin protein
FTH	ferritin heavy chain protein
FTH	ferritin heavy chain gene
FTL	ferritin light chain protein
FTL	ferritin light chain gene
g	gram
G	guanosine
gDNA	genomic deoxyribonucleic acid
GTF (s)	general transcription factor (s)

GGT	gamma-glutamyltransferase
Glu	glutamate
GUSB	beta-glucuronidase protein
GUSB	beta-glucuronidase gene
H (His)	histidine
H^+	proton
H_2O	water
H_2O_2	hydrogen peroxide
HAMP	hepcidin antimicrobial peptide gene
HBC	high burden countries
HCP1	haem carrier protein 1
HEPH	hephaestin protein
HEPH	hephaestin gene
HFE	hemochromatosis gene protein
HFE	hemochromatosis gene
HH	hereditary hemochromatosis
HIF-2α	hypoxia-inducible factor 2 alpha
HIF-1β	hypoxia-inducible factor 1 beta
HIV	human immunodeficiency virus
HMOX1	haem oxygenase-1 protein
HMOX1	haem oxygenase-1 gene
IER (s)	iron exochelin receptor (s)
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
IL-2	interleukin-2
IL-6	interleukin-6
IL-1β	interleukin-1 beta
INH	isoniazid
IRE (s)	iron response element (s)
IRP1	iron regulatory protein 1
IRP2	iron regulatory protein 2
ISR	iron-siderophore reductase
IQR	interquartile range

kb	kilobase
kDa	kilo-Daltons
kg	kilogram
LTF	lactotransferrin protein
LTF	lactotransferrin gene
LRG-47	guanosine triphosphatase
M	moles per litre
mg	milligram
mg/ml	milligrams per millilitre
$MgCl_2$	magnesium chloride
MIQE	minimum information for publication of quantitative real-time PCR experiments
ml	millilitre
mM	millimoles
mRNA	messenger ribonucleic acid
M. tuberculosis	mycobacterium tuberculosis
n	sample size
N	nitrogen
Na	sodium
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
ng	nanogram
ng/µl	nanogram per microliter
NHLS	national health laboratory service
NO	nitrogen oxide
Nramp1	mouse natural resistance-associated macrophage protein 1
Nramp2	mouse natural resistance-associated macrophage protein 2
NRAMP1	natural resistance-associated macrophage protein 1
NRAMP2	natural resistance-associated macrophage protein 2
O_2	oxygen
p	short arm of chromosome

PCA	principle component analysis
PCR	polymerase chain reaction
pH	power of hydrogen
PHD	prolyl-hydroxylase
pmol	picomoles
Pro	proline
PTB	pulmonary tuberculosis
pVHL	von Hippel-Lindau tumour suppressor
PZA	pyrazinamide
q	long arm of chromosome
Q (Gln)	glutamine
qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
R	reverse primer
RBC	red blood cell (erythrocyte)
RIF	rifampin
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
RPL37A1	ribosomal protein L37
RPL37A1	ribosomal protein L37 gene
RT	reverse transcriptase
S (Ser)	serine
S-Albumin	serum albumin
S-FTN	serum ferritin
S-Iron	serum iron
SKP1	s-phase kinase-associated protein 1
SLC11A1	**s**olute carrier family 11, member 1
SLC11A2	**s**olute carrier family 11, member 2
SLC40A1	solute carrier family 40 member 1 gene
SNP	single nucleotide polymorphism
STD	standard deviation
S-Total protein	serum total protein

STF	serum transferrin
T	thymidine
T_A	annealing temperature
TB	tuberculosis
TBE	tris-borate/EDTA
Tf	transferrin
TfR	transferrin receptor
TfR1	transferrin receptor 1
TfR2	transferrin receptor 2
T_M	melting temperature
TNF-α	tumour necrosis factor alpha
TSAT	transferrin saturation
Tris-HCL	tris hydrochloride [2-Amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride]
U	units
UTR	untranslated region
v/v	volume per volume
V	volts
w/v	weight per volume
Y (Tyr)	tyrosine

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PREFACE

The current study aims to provide the reader with an in-depth understanding of the role of iron, the manner in which iron is regulated in the human body and which genes affect iron absorption, storage, export, transport, and recycling. An overview of the research conducted over the past decade linking iron and *M. tuberculosis* infection is subsequently provided. This overview provides the reader with several lines of evidence that support the aim of the current study which is to investigate the correlations that may exist between iron status, iron-gene expression profiles and their association to increased or decreased TB susceptibility. Insight into the pathogenesis of and the immune response to TB is provided to further equip the reader with all the necessary background required to understand the study in totality. Identifying differential iron-gene expression levels that may result in differing levels of susceptibility to TB is investigated using RT-qPCR to generate genetic profiles of eight iron-genes. Correlations between certain genetic profiles and certain iron, liver-, and immune-specific parameters were performed and positive or negative correlations were discussed in terms of TB susceptibility and the interconnected pathway in which these parameters function. A final discussion of the proposed physiological effect of differential gene expression is provided in terms of our current understanding of the iron pathway and increased susceptibility to TB.

Chapter 1

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 IRON

1.1.1 Primary sources and oxidative states

Iron can exist in seven distinct oxidative states; however, the physiological pH in biological systems results in the presence of iron in only two states, namely, ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}) (Pierre *et al.*, 2002). Iron exhibits the ability to bind to several ligands (oxygen, nitrogen and sulphur) because the element can swiftly be converted between these two biological states in a reversible fashion. For successful absorption of this essential element, Fe^{3+} present in dietary sources must be converted to Fe^{2+} at the apical membrane of the duodenal enterocyte (Figure 1). Two distinct sources of iron are available in dietary plant and animal products (Andrews & Schmidt, 2007). The iron present in animal food sources that is incorporated into hemoglobin and myoglobin is termed haem iron while non-haem iron is found primarily in plant food sources. The significant difference between these two types is explained by the rate of absorption in the intestine. Haem iron is readily available for transporter-mediated absorption whereas inorganic (non-haem) iron is taken up *via* the small intestine in a less effective manner (Miret *et al.*, 2003). Interestingly, legumes that contain high concentrations of non-haem iron, which is bound to ferritin (FTN), was previously not described as a source of dietary iron as it was not available for absorption *via* established mechanisms (Lynch *et al*., 1984). A discovery in cultured, Caco-2 human epithelial cells has significantly added to the understanding of iron absorption (San Martin *et al*., 2008). Investigators determined that iron-bound FTN could bind to receptors on the duodenal apical membrane. The iron-FTN complex is engulfed in a clathrin-dependent manner, which allows this complex to enter the duodenal enterocytes. Iron is thereafter liberated from the complex to either be utilised by the cell (export into bloodstream) or stored in the intracellular labile iron pool. The remaining complex is degraded by the enterocyte. Significance and strength has been afforded to these results when data obtained from rat cells and TCMK-1 mouse epithelial cells corroborated these initial findings (Han *et al.*, 2011; Thiel *et al.*, 2012). Iron absorption, storage, export, transport, and recycling are all tightly regulated by several genes and protein products to ensure that the host has absorbed sufficient amounts of iron from the diet to accomplish several crucial functions in the host system on a daily basis.

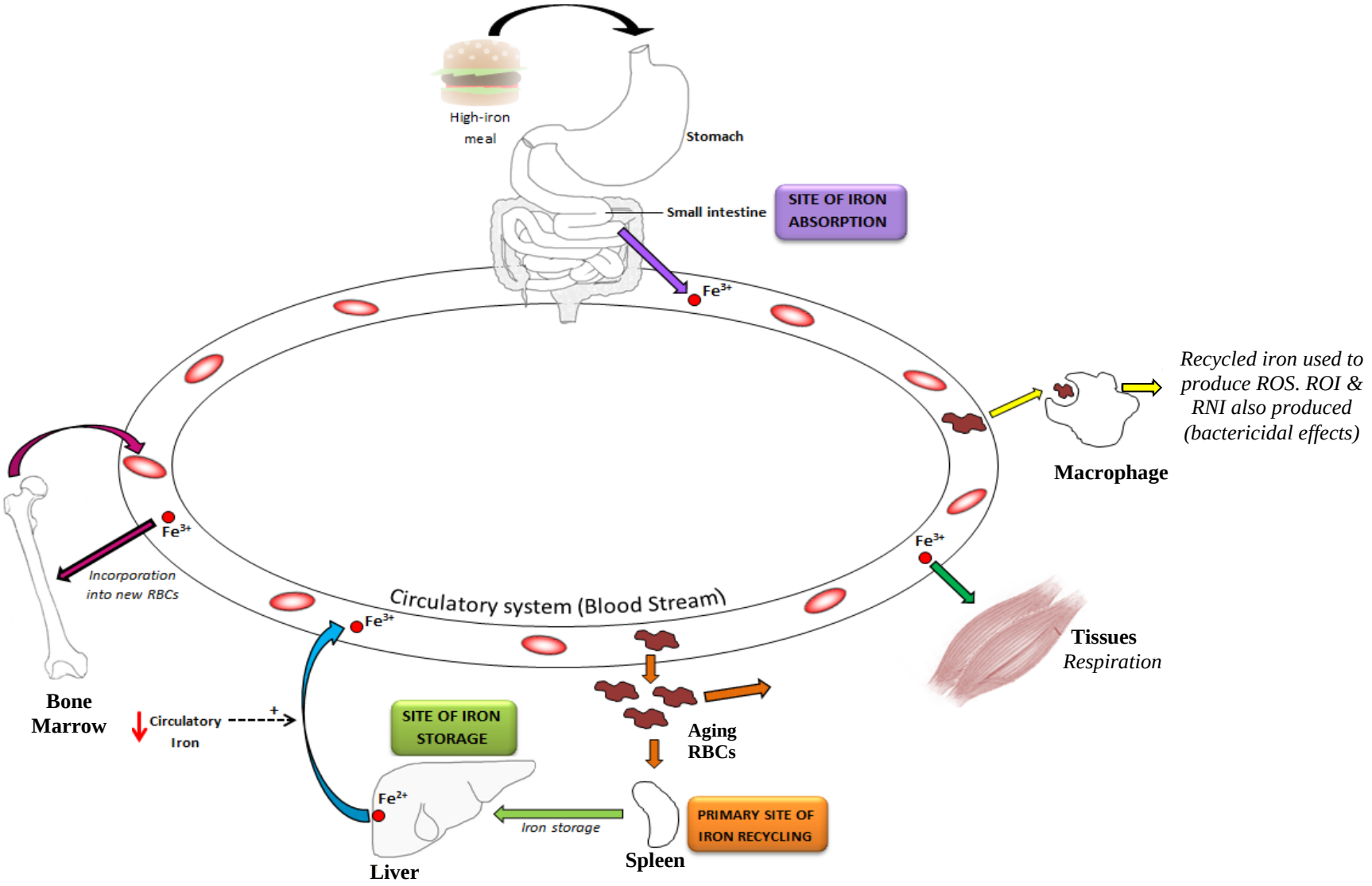

Figure 1 Iron absorption *via* the duodenum and distribution to immune cells, tissues, liver, and bone marrow

Fe^{2+}= ferrous iron; Fe^{3+}= ferric iron; RBCs= red blood cells; ROI= reactive oxygen intermediates; ROS= reactive oxygen species; RNI= reactive nitrogen intermediates

1.1.2 Dietary iron & associated function

The average human body absorbs and secretes an estimated 0.5-2 mg of Fe^{3+} through uptake from the diet and secretion through cell sloughing every day (Andrews, 2000). Fe^{3+} is hydrophobic and therefore requires several protein carriers within the circulatory system to distribute it throughout the body (Anzaldi & Skaar, 2010). The iron content of the human body is 40-50mg Fe/kg and is distributed among haemoglobin (30mg Fe/kg), myoglobin (4mg Fe/kg), haem-, iron-sulphur containing proteins (2mg Fe/kg), transferrin (Tf) (3mg Fe/kg), and FTN (10-12 mg Fe/kg) (Bothwell *et al.*, 1979). Iron-bound FTN is stored within several tissues including immune cells, bone marrow, and liver (Mason & Taylor, 1978) (Figure 1). Upon examination of iron distribution at the physiological level, the highest concentration of Fe^{3+} is present in the haemoglobin molecules of circulating erythrocytes and erythroid bone marrow. The larger percentage of iron allocated to haemoglobin binding is necessary for the imperative binding of oxygen to porphyrin ring structures present in haem molecules (McKie *et al.*, 2001). Erythrocytes subsequently distribute oxygen to a multitude of tissues throughout the body that require it for fundamental processes, including respiration. In addition to the role of iron in oxygen distribution, it also plays a vital role as a cofactor in several processes, including the control of gene expression and cell growth and differentiation (Bothwell *et al.*, 1979; Beard, 2001). Despite the multiple functions that require iron, excess reactive Fe^{2+}, in conjunction with hydrogen peroxide (H_2O_2), has the ability to produce reactive oxygen species (ROS) during redox reactions (Merkofer *et al.*, 2006; Galaris & Pantopoulos, 2008) (Figure 2).

$$Fe^{2+} + H_2O_2 \rightarrow \textit{Reactive Intermediates} \rightarrow Fe^{3+} + {}^{\bullet}HO\,(\text{highly reactive}) + OH^-$$

Figure 2 Formation of active ROS by "Fenton-type" reaction

The buildup of toxic iron and ROS levels occurs as a direct lack of an active excretory mechanism in the human body, which causes damage to healthy tissues (McCance & Widdowson, 1937). Immune cells also produce reactive oxygen and nitrogen intermediates (ROI and RNI) to expedite the chemical breakdown of foreign pathogens as these micromolecules target DNA and several moieties important in cell development (Klebanoff, 1980; Nathan, 1992). The delicate balance of ROS, RNI, and ROI enables the host immune

system to actively combat pathogens in a non-specific, highly-effective manner. The homeostasis of iron, ROS, ROI, and RNI is important as these micromolecules accomplish several important functions and in excess causes damage to several tissues (Latunde-Dada *et al.*, 2004). The primary site of iron regulation is in the duodenal wall which causes alterations in the rate of iron absorption.

1.1.3 Iron Absorption

1.1.3.1 Overview

Iron enters the body when iron-rich plant and animal products are ingested and move through the gastrointestinal tract until it reaches the primary site of absorption: the small intestine (McKie *et al.*, 2001) (Figure 1). The formation of a brush border in the small intestine increases the area of nutrient absorption to facilitate the efficient absorption of a multitude of essentials elements, vitamins, and minerals (Andrews & Schmidt, 2007). Absorption is accomplished by a unique set of trans-membrane transporter proteins that interact with auxiliary enzymes to ensure that Fe^{3+} is reduced to Fe^{2+}, which is the oxidative state required during the process of absorption (Figure 3). Reduction is accomplished by a specific protein called duodenal cytochrome b reductase 1 (DCYTB) (McKie *et al.*, 2001) (Table 1). Once iron is present in the correct oxidative state, it binds to a trans-membrane protein on the duodenal membrane referred to as divalent metal transporter 1 (DMT1/ NRAMP2). Upon binding, Fe^{2+} enters the cell and, if it is not immediately required by the body, binds to the storage protein FTN. An additional protein situated on the duodenal apical membrane that specifically binds to haem iron has subsequently been identified (Shayeghi *et al.*, 2005). The molecule was named haem carrier protein 1 (HCP1) and determined that the entire haem-iron-receptor complex is engulfed by enterocytes. The complex elements that are not required by the cell are degraded, while haem oxygenase 1 (HMOX1) functions to liberate the bound iron for utilization by the cell or subsequent storage. The influx of iron into macrophages is mediated by another protein belonging to the same family, namely, natural resistance-associated macrophage protein 1 (NRAMP1). Macrophages also obtain iron *via* the endocytosis of senescent erythrocytes that are subsequently degraded and iron is liberated by HMOX1 (Beaumont, 2010) (Figure 3). Both pathways of iron absorption in the macrophage are vital in the maintenance of iron stores essential in the host's defence against invading pathogens. In summary, iron absorption by DMT1 and NRAMP1 is initiated when Fe^{3+} is reduced to Fe^{2+} by DCYTB.

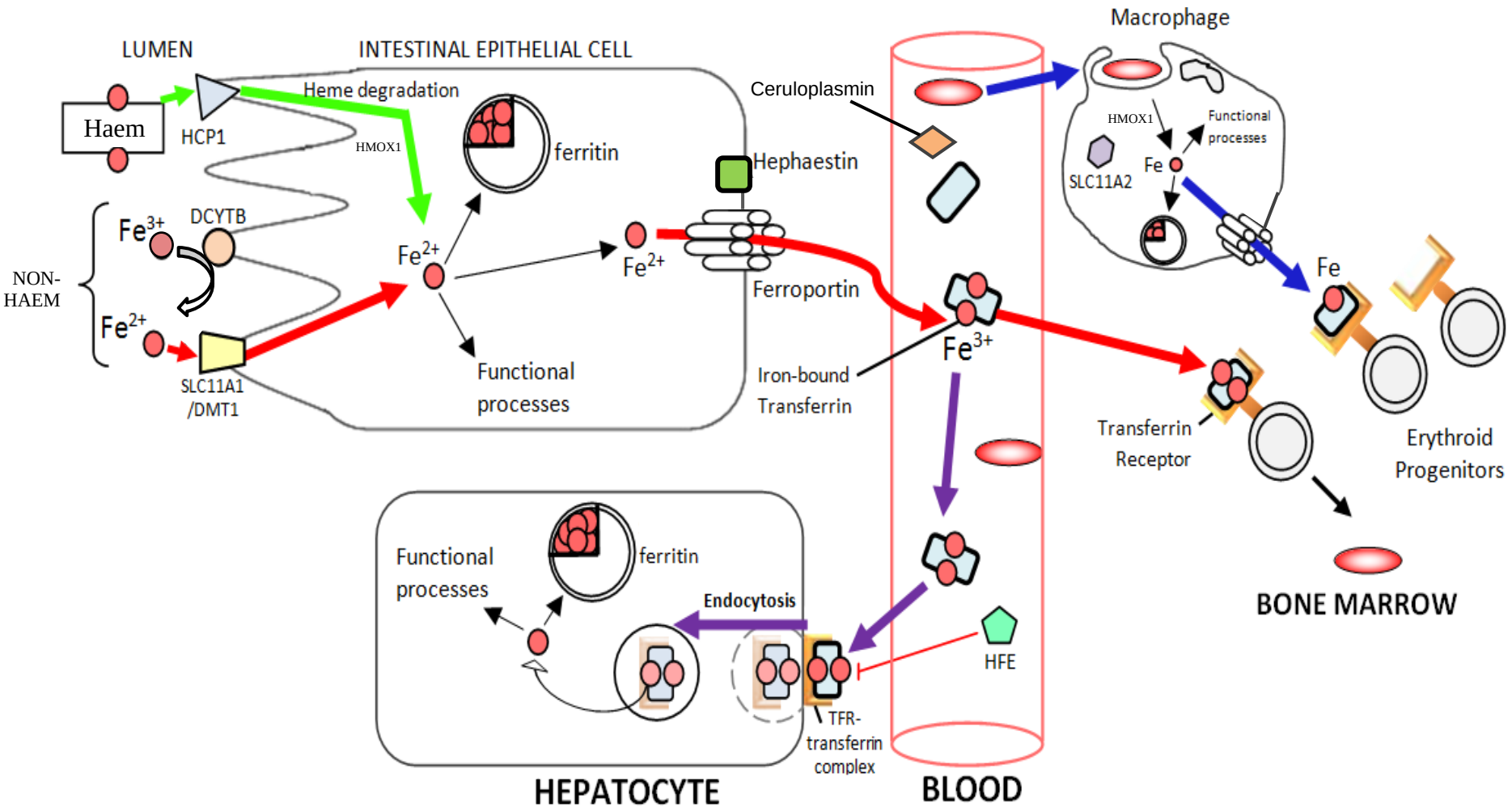

Figure 3 Overall diagram illustrating the absorption, storage, export, transport, and recycling of iron

Iron absorption (left); iron transport into the bloodstream (centre); and recycling in macrophage (right). Iron uptake by hepatocyte (bottom).

DCYTB= duodenal cytochrome b reductase; DMT1= divalent metal transporter 1; Fe^{2+}= ferrous iron; Fe^{3+}= ferric iron; HCP1= haem carrier protein 1; HFE= hemochromatosis protein; HMOX1= haem oxygenase 1; SLC11A1= solute carrier family 11 member 1; SLC11A2= solute carrier family 11 member 2; TfR= transferrin receptor

Table 1 Summary of the genes and proteins that regulate iron homeostasis

GENE				PROTEIN			
Name	Length (bps)	Chromosome position	Number of exons	Name	Length (amino acid residues)	Size (kDa)	General Function
Ceruloplasmin (CP)[2]	59,646	3q23-q25	19	Ceruloplasmin (CP)	1046	132	Oxidise ferrous iron (macrophage & blood steam)
Cytochrome b reductase (CYBRD1)[1]	4365	2q31.1	4	Duodenal cytochrome b reductase 1 (DCYTB)	286	31.6	Reduce ferric iron
Ferritin heavy Chain (FTH)[5]	7,943	11q12.3	4	Ferritin heavy Chain subunit (FTH)	183	21.2	Subunit of iron storage molecule
Ferritin light Chain (FTL)[5]	1,571	19q13.33	4	Ferritin light Chain subunit (FTL)	175	20	Subunit of iron storage molecule
Hepcidin antimicrobial peptide (HAMP)	2,637	19q13.1	3	Hepcidin antimicrobial peptide (HAMP)	84	9.4	Regulates iron homeostasis *via* ferroportin internalization
Hephaestin (HEPH)[3]	106,319	Xq11-q12	21	Hephaestin (HEPH)	1158	130.4	Oxidises ferrous iron (intestine-bloodstream barrier)
Haemochromatosis gene (HFE)	11,124	6p21.3	7	Haemochromatosis protein (HFE)	348	40.1	Regulates iron absorption *via* Tf receptor binding
Haem oxygenase 1 (HMOX1)[4]	1822	22q12	5	Haem oxygenase 1 (HMOX1)	288	32.8	Cleaves haem to ultimately release iron (haem recycling)
Lactotransferrin (LTF)	49,589	3p21.31	17	Lactotransferrin (LTF)	710	78.1	Immune response & iron transporter
Ferroportin (FPN/ SLC40A1)[3]	23,180	2q32.3	8	Ferroportin (FPN/ SLC40A1)	571	62.5	Exports iron from within intestinal/ macrophage cells to bloodstream
Solute Carrier family 11, member 1 (SLC11A1)[1]	14,866	2q35	15	Natural Resistance-Associated Macrophage Protein 1 (NRAMP1)	550	59.8	Macrophage iron absorption & pathogen resistance
Solute Carrier family 11, member 2 (SLC11A2)[1]	49,166	12q13	16	Natural Resistance-Associated Macrophage Protein 2 (NRAMP2)	568	62.2	Duodenal iron absorption

[1] absorption; [2] transport; [3] export; [4] recycling; [5] storage

1.1.3.2 Iron reduction in gut lumen

The highest level of iron acquisition takes place at the upper villus region (brush border) of the duodenum where the largest amount of fully developed enterocytes is located (O'Riordan *et al.,* 1995). Research published in 1995 concluded that Fe^{3+} is reduced to Fe^{2+} on the apical membrane of duodenal enterocytes by a protein present on the enterocyte membrane (Riedel *et al.,* 1995). McKie *et al.* (2001) later determined that this isolated protein is also expressed in the region of the intestine where the highest level of iron is absorbed and therefore named the protein duodenal cytochrome B (DCYTB). Results were confirmed in 2008 when researchers determined that iron absorption is stimulated by DCYTB expression in human intestinal cells (Latunde-Dada *et al.,* 2008). A degree of homology was found between this ferrireductase protein and the cytochrome b561 in sheep during an initial Basic Local Alignment Search Tool (BLAST) search (Okuyama *et al.,* 1998). Researchers determined that four histidine (His) residues were conserved when the sequence was aligned to the b561 sequences of various species, including mouse and bovine. The investigators also showed that the four His residues found in the b561 sequence exhibited the ability to bind to haem. Due to the significant level of homology between DCYTB and b561 across several species, McKie *et al.* (2001) predicted that the His residues in DCYTB bound haem as a ligand and therefore acts as the target protein responsible for Fe^{3+} reduction. Another research group demonstrated that iron deficiency significantly induced *cytochrome b reductase 1* (*CYBRD1*) expression, thereby signifying its pivotal role in iron homeostasis (Oakhill *et al.,* 2008) (Table 1). Further investigation reported increased expression of the protein to the magnitude of five- to six-times when Dcytb cRNA-injected *Xenopus* oocytes were compared to *Xenopus* oocytes that were treated with water (mock version). The research group verified these findings when significantly elevated reductase activity was obtained after the transfection process in human cell lines (HuTu-80 and CaCo-2 cells). Although DCYTB has been described as the ferrireductase responsible for the Fe^{3+} required for absorption, Fe^{2+} needs to bind to a divalent cation trans-membrane protein to enter the mucosa of the duodenum. Several proteins localised to the macrophage membrane (NRAMP1) and the apical border of the intestine (NRAMP2/ DMT1 and HCP1) has been identified as the chief import proteins that facilitate iron absorption.

1.1.3.3 Several gene products responsible for absorption

A candidate gene associated with susceptibility to mycobacterial infection in mice, then referred to as *Bcg*, was identified in 1982 (Skamene *et al.*, 1982). Later referred to as *NRAMP1* (Vidal *et al*., 1993), the encoded protein (NRAMP1) influences the ability of host macrophages to regulate the proliferation rate of microbes throughout the initial response to infection before the innate immune response commences. The protein functions as a proton (H^+)/divalent cation metal antiporter positioned on the macrophage membrane to enable cation movement during host response to mycobacterium infection (Blackwell *et al.*, 2000). A research group identified and characterised a mouse *Nramp2* gene, also known as *Dmt1*, which shared 78% amino acid homology to *Nramp1* (Gruenheid *et al.*, 1995). The chromosomal position of *DMT1* was localised to chromosome 12 (Vidal *et al.*, 1995). Both protein products were known to play a role in susceptibility to mycobacterial infection, however, the specific function of NRAMP2/DMT1 was unclear until a study was published in 1997 (Gunshin *et al*., 1997). The investigators determined that NRAMP2 acts as H^+-coupled metal ion-transporter that is expressed in most tissues, with the highest expression observed in the duodenum. During periods of iron-deficiency, NRAMP2 is up-regulated to increase the influx of iron into the enterocytes and, therefore, acts as a primary regulator of iron homeostasis in the host organism. In 2005, researchers purified a membrane-bound transporter protein highly expressed in the proximal region of the intestine responsible for haem import from the lumen (Shayeghi *et al.*, 2005). During further investigation of HCP1 in iron deficiency, the protein demonstrated sensitivity towards iron levels. The researchers concluded that the protein is sensitive to iron status and, therefore, plays an important role in iron homeostasis *via* haem-iron absorption. Apart from iron storage, iron absorption through DCYTB-mediated reduction and NRAMP2 import serves as the key process that can be altered to maintain iron balance in the body, thereby reducing oxidative damage caused by excess iron concentrations (Alkhateeb & Connor, 2010).

1.1.4 Storage of Iron

1.1.4.1 Overview

Excess iron that is not immediately required by the body is bound to FTN and stored until the iron is needed by the different tissues in the body for vital functioning. Excess iron present in a cell results in the production of reactive species *via* Fenton reactions (Figure 2), which ultimately lead to lipid, DNA, and protein damage and a significant increase in cellular

toxicity (Torti & Torti, 2002; Merkofer *et al.*, 2006; Galaris & Pantopoulos, 2008). FTN serves to detoxify the cellular environment from excess iron that may become harmful if not bound and stored in the labile iron pool (Boyd *et al.*, 1984; Mattia *et al.*, 1986). FTN is a highly conserved protein consisting of 24 light- (FTL) and heavy-chain (FTH) subunits and is primarily present in the spleen, liver, bone marrow, and macrophages but is likely to be present in small amounts within every cell of the body (Boyd *et al.*, 1984; Harrison & Arosio, 1996). The 24 subunits collectively form the apoferritin protein, which is 450kD in size and exhibits the ability to sequester a total of 4500 Fe^{2+} ions. Fe^{2+} undergoes several oxidative conversions to produce the non-reactive Fe^{3+} form that is stored within the nano-cavities that form in the centre of a 24-subunit apoferritin. Fe^{2+} binds to a catalytic ferrioxidase site in FTN where the process of oxidation commences. Several intermediates are produced to ultimately produce Fe^{3+} (Liu & Theil, 2004). The ferroxidase centre is a highly conserved sequence with the His65 and glutamine (Glu) 62 mutations leading to a drastic reduction in activity and overall FTN function (Lawson *et al.*, 1989). Due to the protein's size and its high binding affinity for iron, it is highly suited as the primary iron-storage protein in the body (Harrison & Arosio, 1996).

1.1.4.2 Genes responsible for iron storage and their regulation

Regardless of the widespread sequence homology between FTL and FTH, the proteins are encoded by two entirely different genes - ferritin light chain (*FTL*) and ferritin heavy chain (*FTH*) respectively (Watanabe & Drysdale, 1981; Hentze *et al.*, 1986) (Table 1). Several research groups were instrumental in the localization and characterization of both FTN subunits (Caskey *et al.*, 1983; McGill *et al.*, 1984; Worwood *et al.*, 1985). The H-chain is distinct from the L-chain as it possesses the ability to oxidise Fe^{2+} to Fe^{3+}, which facilitates the binding of the element and its subsequent storage within the labile pool (Lawson *et al.*, 1989; Levi *et al.*, 1992). The protein level within cells significantly increases as the amount of absorbed iron increases, indicating that FTN levels are highly regulated by iron status (Rittling & Woodworth, 1984). Two independent groups determined that iron-mediated FTN regulation occurs at the transcriptional and the translational levels, demonstrating that the study of how *FTL* and *FTH* expression is altered during different iron states (high, medium, and low levels) is an important aspect of the current study (Cairo *et al.*, 1985; White & Munro, 1988).

In terms of regulation at the translational level, the iron regulatory proteins (IRP1 and IRP2) control the production of both the heavy and light chains of FTN during iron abundant (increase translation) and iron deficient (decrease translation) conditions. Control is achieved when an IRP binds to iron responsive elements (IREs) situated in the 5'- untranslated regions (UTR) of both the heavy and light ferritin mRNA molecules (Leibold & Munro, 1988; Eisenstein, 2000). Transcriptional regulation is accomplished *via* factor (tumour necrosis factor-α (TNF-α); interluekin-1β (IL-1β) and insulin-like growth factor-1 (IGF-1)) binding to the H-subunit, which affects the H-subunit to L-subunit ratio. Increased H-subunit levels are vital within organs that require a high turnover of iron for functional processes, while iron-storage organs such as the liver exhibit higher levels of FTL. Torti and Torti (2002) reviewed a vast spectrum of articles in which numerous researchers demonstrated that changes in the L-to-H subunit ratio not only influenced iron homeostasis but deviations were also seen during certain infectious and inflammatory diseases.

1.1.4.3 Additional functions of FTN

A study conducted in 2000 demonstrated that the homozygous knockdown of *FTH* in murine is lethal (Ferreira *et al.,* 2000). These findings indicate that the H-subunit of FTN plays an important role during embryonic development by acting as a powerful anti-oxidant. FTH also exhibits an affinity for several signalling elements that form part of survival pathways. The heavy chain subunit was documented as a negative regulator of CXC ligand 12 (CXCL12) that binds to surface bound CXC chemokine receptor 4 (CXCR4). Research then continued on the protein's interactions with CXCR4 and it was discovered that FTN was being translocated to the nucleus (Alkhateeb & Connor, 2010). Nuclear translocation occurred when cells were exposed to CXCL12 and when FTN was phosphorylated in a time-dependent manner. Due to the protective role FTH plays within the cellular system, the nuclear translocation of FTH indicates that it may be vital in the protection of DNA against H_2O_2- and ultraviolet light- induced damage (Cai *et al.,* 1998; Cai *et al.,* 2008). These findings were confirmed when a study indicated that FTN (FTH subunit) has the ability to bind to DNA and might function as a transcription factor regulating transcriptional processes (Broyles *et al.,* 2001). Researchers demonstrated that spleen FTN antibodies prevented the production of a protein-DNA complex that generally forms when nuclear extracts from *K562* cells were incubated along with a certain region of the *β-globin* distal promoter. The complex showed several properties of FTN, including thermo-stability and proteinase K resistance. Heart and liver FTN molecules also displayed similar affinity for the identified *β-globin* promoter

region (CAGTGC). Mutation based studies that altered the identified recognition sequence counteracted the repression of the *β-globin* promoter when co-transfected with both the heavy and light FTH forms in cultured cells. Only heavy-chain subunit displays the ability to bind the identified region, suggesting that the Ferroxidase activity specific to FTH affects DNA binding (Surguladze *et al.*, 2004). FTN's ability to bind DNA is independent of length, sequence, and base composition, therefore allowing the protein to be within close proximity to DNA at all times to facilitate binding and transcription factor activity. The identification and acceptance of FTN as a possible transcription factor requires further investigation, although the ground work and initial evidence is promising. Various researchers have demonstrated the significant role FTN plays within iron homeostasis and overall organism survival, which validates the study of *FTL* and *FTH* gene expression differences between healthy and tuberculosis (TB) infected individuals in relation to increased susceptibility to infection. To enable iron storage in primary organs, this element must enter into the enterocyte and, subsequently, binds to certain carrier proteins in the bloodstream.

1.1.5 Iron Export & Transport

1.1.5.1 Overview

Since iron plays a pivotal role in multiple pathways and is important for the functioning of a vast array of enzymes, the element is needed by most of the cells throughout the body. Since iron is primarily absorbed in the villus of the intestine, the element needs to pass through the enterocytes into the bloodstream and then travel to the desired destination. For iron to be exported from the enterocytes, it needs to be converted to Fe^{3+}. This reaction is catalysed by the oxidation of Fe^{2+} by hephaestin (HEPH) and subsequent transfer into the bloodstream through ferroportin (FPN) on the membrane. HEPH exhibits the required ferroxidase activity to allow export *via* FPN. Another ferroxidase is present in the plasma and facilitates the oxidation of Fe^{2+}. The soluble form of iron subsequently binds to apotransferrin or lactotransferrin (LTF) molecules present in the plasma and bodily secretions. Tf and LTF are responsible for the transport of iron to the sites throughout the body that require it for vital functioning.

1.1.5.2 Primary gene responsible for export and its interactions

FPN was first identified by the name metal transport 1 (MTP1) (Abboud & Haile, 2000). It is situated at the plasma membrane to facilitate its all-important function of iron export from

within enterocytes, macrophages, Kupffer cells, placental cells, and brain astrocytes (Beaumont, 2010) (Figure 3). The functioning of this protein is mediated by the presence of a ferroxidase molecule, either HEPH, present in the enterocytes, or ceruloplasmin (CP), present in the plasma (Figure 3). These proteins are fundamentally required to oxidise Fe^{2+} to Fe^{3+}, which allows the release of iron into the circulatory system. Since FPN is the only known exporter of iron, it plays an essential part in the release of iron obtained through haem degradation during erythrocyte recycling within tissue macrophages (Beaumont, 2010). FPN is encoded by the solute carrier family 40 member 1 gene (*SLC40A1*), otherwise referred to as the ferroportin gene (*FPN*), and was localised to chromosome 2 (Haile, 2000). The protein passes through the plasma membrane a multitude of times to ensure that intracellular iron is released into the circulating plasma (Rice *et al.,* 2009) (Figure 4). Since FPN is presently the only known iron-exporter protein, differential expression of *FPN* would be expected in the incidence of individuals with elevated susceptibility to developing active TB because the invading pathogen requires available iron to survive. Previous research demonstrated that lower *CYBRD1* expression levels result in reduced protein product levels leading to an increase in intracellular iron since the element cannot escape the cell (Collins, 2008). Moreover, *FPN* expression levels are regulated by the synthesis and release of hepcidin from the liver when iron levels are elevated in the body (Park *et al.*, 2001; Nemeth *et al,* 2004). Once hepcidin has bound to FPN, the iron exporter is phosphorylated and ubiquitinated, resulting in its subsequent internalization and ultimate inactivation (De Domenico *et al.,* 2007).

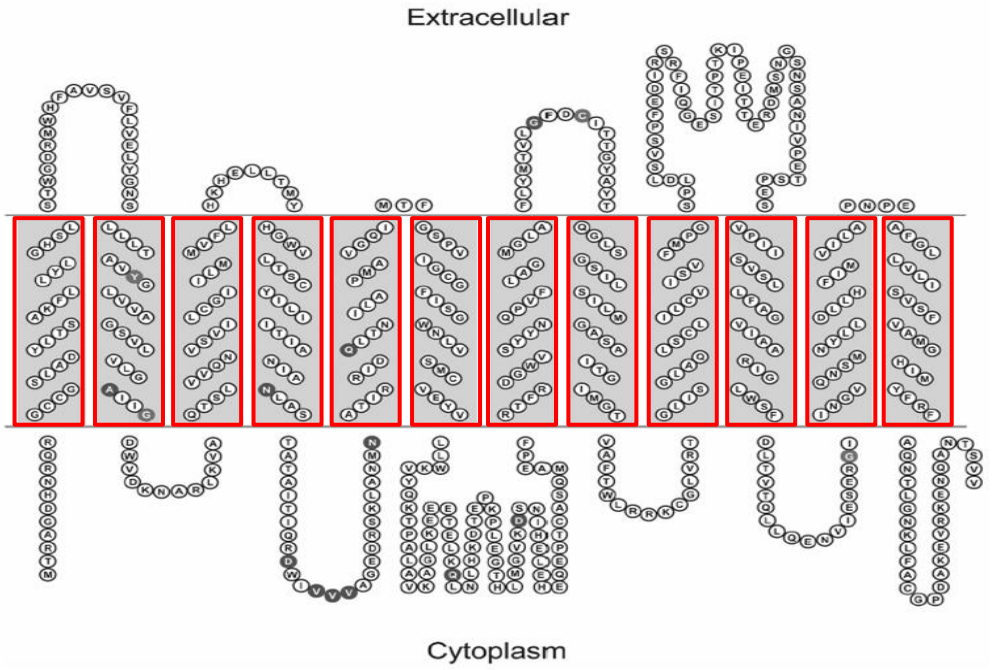

Figure 4 Structure of FPN demonstrating several regions (red) of the transverse protein

Adapted with permission from Rice (2009)

1.1.5.3 Oxidation of iron required for export

HEPH and CP are primarily responsible for the oxidation of Fe^{2+} to Fe^{3+}, which facilitates iron export out of enterocytes and macrophages and/or transport in the blood stream. Although both proteins exhibit ferroxidase activity, they differ significantly in terms of localization. HEPH is membrane-bound and found at the interface between enterocytes/ macrophages and plasma to accomplish successful iron export directly into the blood flow (Han & Kim, 2007). CP is produced in the liver and released into the circulatory system to bind and, subsequently, oxidise ferrous iron to allow ferric iron to bind to Tf molecules (iron transporter within the blood) (Neifakh *et al.,* 1969). HEPH is encoded by the hephaestin gene (*HEPH*) and has the ability to bind 6 copper (Cu) atoms. The ceruloplasmin gene (*CP*) encodes for CP, which has the ability to bind to the Cu atoms which are present in the blood-steam (Takahashi *et al.*, 1984; Yang *et al.,* 1986). Cu is required by iron to perform vital molecular functions and is furthermore required by the body as an inherent defence mechanism against the damage caused by ROS (Uriu-Adams & Keen, 2005). These two essential elements are linked through CP to achieve the protein's ferroxidase activity (Cernat *et al.,* 2011). Due to the significant role iron and Cu play within the defence against invading pathogens—including inflammation—the level of both elements are reduced during active infection to lessen bioavailability to the developing bacilli (Karyadi *et al.,* 2000). Higher

levels of CP and Cu were demonstrated in patients with active pulmonary tuberculosis (PTB) in a recent study (Cernat *et al.*, 2011). Findings from a previous study demonstrate that patients who presented with aceruloplasminemia (hereditary CP deficiency) accumulated excess amounts of iron within tissues (Mukhopadhyay *et al.*, 1998). These findings further signify the importance of CP in the regulation of iron balance and that the protein plays a vital role in the release of iron from tissues. Although iron has the ability to be exported out of the tissues, it must be bound to a transporter protein (either LTF or Tf) in the bloodstream to be distributed.

1.1.5.4 LTF and Tf proteins are responsible for transport

During the initial identification and characterization of Tf proteins, it was found that they exhibit antimicrobial properties. These properties were demonstrated when researchers reported that the proteins acted as a defence mechanism against bacterial infection by decreasing the availability of iron (essential to bacterial proliferation) and thus contributing to overall defence in humans. This family of proteins is often separated into several groups according to their individual functions and locality. A Tf that is manufactured by the liver and thereafter released into the blood stream is referred to as serum-transferrin (STF). The primary function of this group of Tfs is to transport iron from the absorption and haem degradation sites to the areas throughout the body that require the essential element for vital functions. The effectiveness of iron transportation is dependent on the amount of iron present within the bloodstream.

The transferrin gene (*TF*) was localised to chromosome 3; and regions that bind iron remain conserved during evolution, illustrating the importance of Tf in iron transport (Yang *et al.*, 1984). Sorensen and Sorensen (1939) were the first to discover a novel protein present in milk collected from cattle. After its discovery, the protein was isolated from bovine milk by three independent research groups and was appropriately named LTF (Groves, 1960; Johanson, 1960; Montreuil *et al.*, 1960). The similarity found between LTF and Tf was documented as the protein turned red while it was incubated with ferric iron, similar to when Tf and ferric iron are incubated. Following its discovery, LTF was uncovered in surface mucosa epithelium, secondary granules of neutrophils, and a variety of alternative bodily secretions. The protein is encoded by the *lactotransferrin gene* (*LTF*) and belongs to the specific Tf gene family. This particular protein family encode for proteins that are situated in the secondary granules of neutrophils (Masson *et al.*, 1969; Baggiolini *et al.*, 1970). The gene

has been localised to chromosome 3 and the amino acid structure of LTF has been determined (Metz-Boutigue *et al*., 1984; Naylor *et al.*, 1987; Kim *et al*., 1998). Metz-Boutigue (1984) determined that the protein contains two sites that specifically bind metals, including Fe^{3+}. LTF was initially characterised by its ability to sequester iron but later became known for its role within the immune system since mice infected with TB showed elevated levels (Welsh *et al.*, 2011).

In addition to the protein's role in iron sequestrating and the immune system, LTF has demonstrated several anticancer characteristics (Legrand *et al.*, 2008). The presence and availability of iron is imperative for the proliferation of *Mycobacterium tuberculosis* (*M. tuberculosis*) during the infectious cycle of the bacterium. Results obtained by a group in 2002 showed a significant reduction in the burden of infection when beta-2 microglobulin (β2m) deficient mice were treated with an exogenous source of LTF since the protein could bind iron and reduce its availability to the pathogen (Schaible *et al.*, 2002). Upon infection, the immune system initiates an inflammatory response in the coordinated attempt to destroy the pathogen. LTF acts as a competitive binding protein to sequester iron in the plasma to decrease the availability of this essential element to the pathogen (Korbel *et al*., 2008). Low levels of the protein causing an noted elevation in free iron, which intensifies the proliferation of the bacterium. *LTF* expression is expected to be low in those individuals that demonstrate an increased susceptibility to TB (Collins, 2008). Two studies demonstrate that *LTF* is differentially expressed among different tissues, as well as different species (Shigeta, 1996; Grant *et al.*, 1999). Moreover, *LTF* expression fluctuates within the endometrium, with an augmentation in levels throughout the proliferative phase of the female menstrual cycle and a decrease during the lateal phase (Teng, 2002). Due to increased estrogen levels within this specific menstrual phase, studies that measured *LTF* expression showed that expression was up-regulated by estrogen in the uterus. The amount of iron available for transport is controlled by the rate of iron absorption, which in turn is controlled by both the hemochromatosis gene (*HFE*) and hepcidin antimicrobial peptide (*HAMP*).

1.1.6 Regulators of iron homeostasis

1.1.6.1 Regulation of iron export

In 2001, two independent research groups focused on identifying the protein responsible for iron overload and determining its expression in murine animals (Park *et al.*, 2001; Pigeon *et*

al., 2001). Differential expression of hepcidin was seen in the liver of these murine animals and researchers therefore postulated that the protein functions within the complex iron pathway in these animals (Pigeon *et al.*, 2001). A research group demonstrated that the protein has distinct anti-bacterial properties (Park *et al.*, 2001). Furthermore, once the bacterium has infected the host, interleukin-1 and 6 (IL-1 and IL-6) stimulate the liver to produce this protein product. Protein product levels are also inversely correlated with the export of iron from macrophages, further denoting the protein's important role as an antibacterial agent (Fleming & Sly, 2001; Ganz, 2003). Hepcidin is encoded by *HAMP* and is also required for the efficient storage of iron within macrophages (Jordan *et al.*, 2009). Hepcidin levels have been shown to inversely correlate with the pace at which iron is taken up in the intestine. Hepcidin accomplishes its functional role in the control of iron export through FPN degradation on the basolateral membranes of enterocytes (Nemeth *et al.*, 2004) (Figure 5). An increased concentration of this protein results in elevated intracellular iron levels and diminished plasma iron levels because iron cannot escape the cells that store iron (Ganz, 2003). In addition, an inverse relationship exists between hepcidin expression levels and iron absorption in the intestine and iron export from erythrocyte recycling in splenic macrophages (Nicolas *et al.*, 2002; Ganz, 2005).

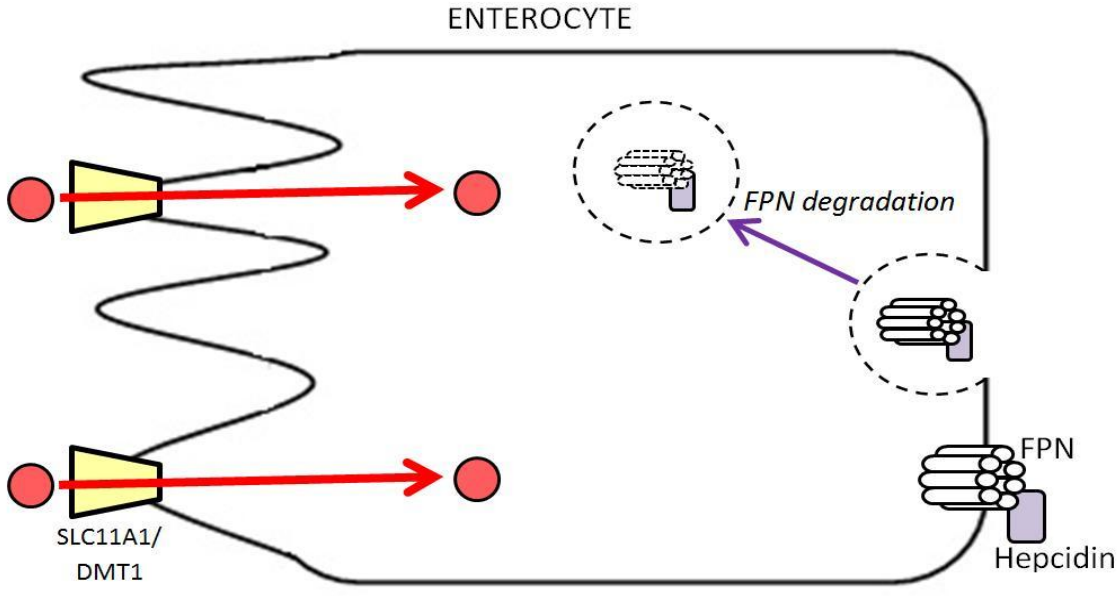

Figure 5 Hepcidin binds to FPN on the basolateral membrane of enterocytes and causes FPN internalization and ultimate degradation

DMT1= divalent metal transporter 1; FPN= ferroportin protein; SLC11A1= solute carrier family 11, member 1

Adapted with permission from Ganz (2005)

Although hepcidin expression actively regulates iron homeostasis, several factors, including oxygen, iron, and chemokine (IL-6) levels, directly influences the secretion rate of hepcidin (Nicolas *et al.*, 2001). Increased hepcidin levels that result in plasma hypoferremia are ideal for defence against bacteria, specifically those that target the gut, as iron is withheld from these pathogens in an attempt to decrease iron bioavailability. However, this may be detrimental in the case of *M. tuberculosis* infection as the bacterium is engulfed by the macrophages where iron stores are now high. In effect, hepcidin and its iron withholding properties may provide the perfect environment for *M. tuberculosis* proliferation. Overall, it is anticipated that individuals with an elevated susceptibility to TB infection may exhibit increased expression of hepcidin (Nicolas *et al.*, 2003; Collins, 2008).

1.1.6.2 Regulation of iron absorption

Discovered in 1996, the *HFE* gene that codes for the hemochromatosis protein (HFE) regulates iron absorption *via* the transferrin receptor (TfR)-Tf receptor complex on the hepatocyte and macrophage membranes (Feder *et al.*, 1996). This function is accomplished through protein binding to TfR, which subsequently decreases the receptor's binding affinity for its intended ligand, Tf (Figure 3). Schaible *et al.* (2002) demonstrated that β2m binds to HFE (major histocompatibility complex I-like protein) preventing its association with TfR thereby allowing the free movement of iron (TfR-Tf complex) into the cell. Three primary mutations (H63D, C282Y and S65C) within this gene have been linked to Hereditary Hemochromatosis (HH), a disorder denoted by the build-up of harmful levels of iron within the cells that can become toxic to the cells (Feder *et al.*, 1996; Barton *et al.*, 1999). Although the disease was initially documented in 1889, the underlying genetic basis thereof has only been discovered and examined in the past decade.

1.1.7 Iron recycling

1.1.7.1 Role of HMOX1 in iron recycling

Iron that is taken up *via* the duodenum and bound to Tf accounts for a relatively small portion of the element present in the circulatory system. Iron derived from the breakdown of damaged erythrocytes provides the most significant amount of iron to the circulatory system as the body lacks of an active mechanism to excrete excess iron and iron absorption only contributes 0.1% to the entire iron status (Andrews & Schmidt, 2007). This enzyme is responsible for the cleavage of haem to form biliverdin. Biliverdin reductase thereafter

converts biliverdin to bilirubin (powerful antioxidative properties) with the liberation of carbon monoxide (CO) and iron (Stocker *et al.*, 1987) (Figure 6). Chung *et al.* (2008) demonstrated that HMOX1 null mice were at a significantly higher risk of polymicrobial infection. High expression is observed in the spleen as it acts as the primary site for the sequestration and degradation of senescent erythrocytes (Figure 1). *HMOX1* is induced when haem concentrations are high during physiologically stressful situations (Kutty *et al*., 1994). Haem acts as the prosthetic group for several enzymes and has the ability to bind oxygen to facilitate its movement (Lu *et al.*, 2001). Work on *Streptococcus agalactiae* shows that a decrease in virulence is noted when intracellular levels of haem are low (Fernandez *et al.*, 2010). Alternatively, *Staphylococcus aureus* hypervirulence was documented in a mouse model after haem levels were significantly elevated (Torres *et al.*, 2007). Overall, the regulation of haem biosynthesis and catabolism is necessary for the optimal functioning of both the host and the bacterium. It is categorised as a cyto-protective enzyme that acts to protect the system against the harmful effects that accompany an inflammatory response to infection (Poss & Tonegawa, 1997).

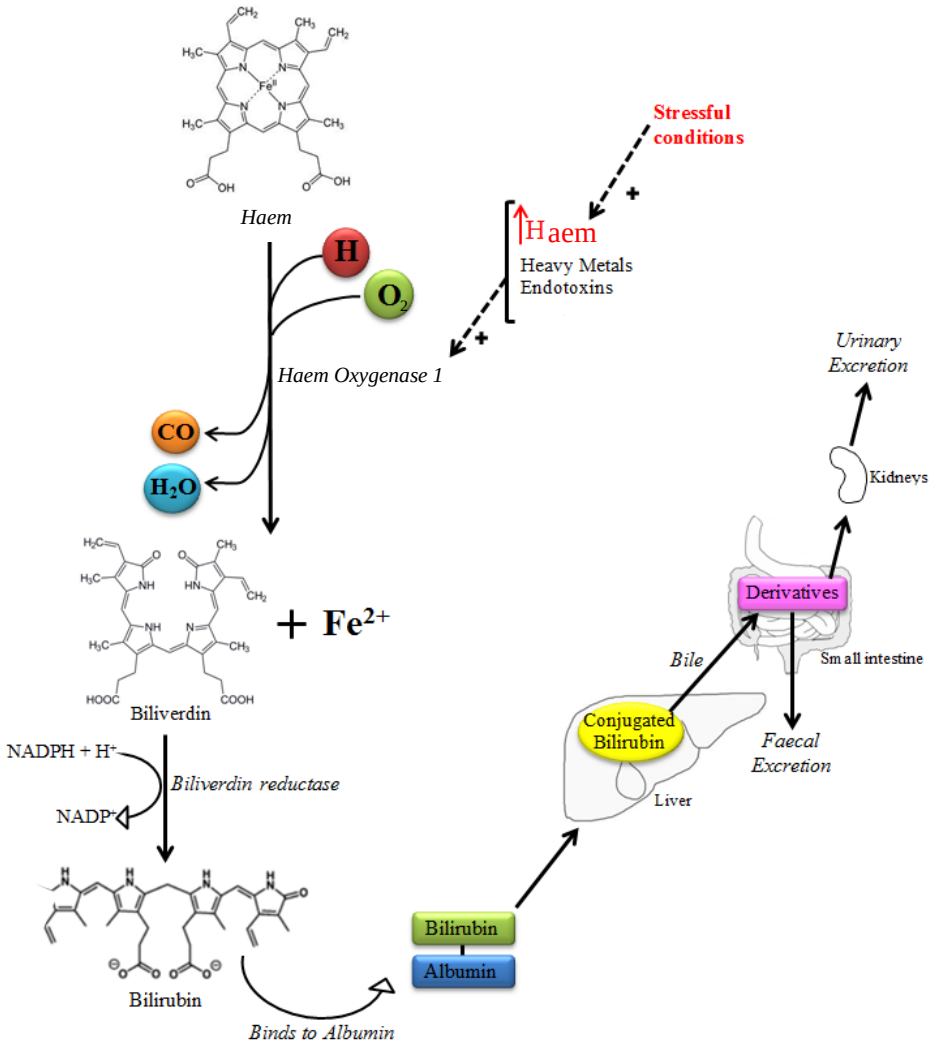

Figure 6 Iron recycling catalysed by haem oxygenase 1 and excretion of bilirubin (reaction by-product)

CO= Carbon monoxide; H= hydrogen; H^+= proton; H_2O= water; $NADP^+$= nicotinamide adenine dinucleotide phosphate; NADPH= reduced from of nicotinamide adenine dinucleotide phosphate; O_2= oxygen

1.1.8 Iron abnormalities

1.1.8.1 Disease States

Several disease states may develop as a result of iron dysregulation caused by mutations that occur in genes that function within the complex iron pathway. HH is an autosomal recessive disorder and is the most frequently occurring (1 in every 200-300 individuals) disease state that results from polymorphisms identified within the *HFE* gene (Simon *et al.*, 1976; Feder *et al.*, 1996; Barton *et al.*, 1999). HH occurs at a higher frequency in males when compared to females and is most often reported within white populations of a Northern European descent

(Phatak *et al.,* 1998; Milman *et al.,* 2003). Symptoms such as fatigue, weight loss, arthritis, diabetes, and hyperpigmentation result from an increased absorption of iron by the small intestine, which subsequently accumulate in the heart, liver, and pancreas causing organ damage. Another form of hereditary iron overload inherited in a dominant manner, referred to as ferroportin disease, shares common traits with HH (Pietrangelo *et al*., 1999). Several distinguishing factors were reported when C282Y-associated HH and the newly discovered form of hemochromatosis were compared. Features included an early decrease in serum-iron and haemoglobin levels in comparison to FTN concentration during therapeutic phlebotomy, intolerance to therapeutic phlebotomy, and early augmentation in serum-FTN levels when transferrin saturation (TSAT) percentage was low. The most distinctive difference is noted in the localization of iron accumulation, which occurs in the Kupffer cells, rather than in the hepatic, heart and spleen tissues (Pietrangelo, 2003). The phenotypic manifestations of ferroportin diseases are similar to those documented for Bantu-siderosis (later referred to as African iron-overload) (Bothwell *et al.*, 1964). Mutation screening of *SLC40A1* delivered a non-synonymous single nucleotide polymorphism (SNP) (Q248H) that was found in a high frequency in southern African individuals presenting with elevated iron levels (Gordeuk *et al.,* 2003). This finding provides preliminary evidence that *SLC40A1* exhibits a modifier activity in African iron overload. The A49U mutation was identified in *FTH* which is associated to a less prevalent form of iron overload called H-ferritin-associated HH (Kato *et al.,* 2001).

1.1.8.2 Testing for iron-overload induced liver damage

Toxic levels of iron found within the liver is a common occurrence among individuals suffering from iron overload (Pratt, 2010). To determine the liver damage as a possible result of toxic iron levels, a panel of blood tests can be prescribed by a practitioner for an individual suffering from iron overload. Often incorrectly referred to as liver-function tests, the panel consists of several enzymes related to liver status, including alanine aminotransferase (ALT), alkaline phosphatase, aspartate transaminase (AST), gamma-glutamyltransferase (GGT) (otherwise known as γ-glutamyl transpeptidase- GGTP), and bilirubin (total, un-conjugated and conjugated).

1.2 GENE REGULATION

The coordinated, time-specific control of gene and protein expression is of vital importance in the accurate development of every living organism (Goodrich *et al.*, 1996). The

mechanisms that are employed by eukaryotic organisms to regulate the expression of certain genes during several stages of development or within pre-determined tissue types have been under investigation for decades (Maniatis, 1987). Progress has been made in recent years using statistical algorithms combined with mRNA data (Tavazoie *et al.*, 1999) or gene expression profiles (Bussemaker *et al.*, 2001) to identify specific *cis*-acting sequences that regulate gene expression. Numerous proteins that bind to these *cis*-acting elements have been identified and these protein-DNA interactions that result in gene regulation have become an important topic of investigation (Bolouri & Davidson, 2003). For the successful transcription of eukaryotic genes, several elements localised to the upstream or downstream regions of exonic gene regions must be activated. Promoters, forming the first category of gene-regulating elements, are positioned up-stream of the start codon and specifically result in the initiation of the transcriptional process. Several general transcription factors (GFTs) act in tandem with RNA polymerase to identify and bind to specific recognition sites upstream of exonic regions to aid in the process of transcription. GFTs include transcription factor II- A, B, D, E, F, and H that facilitate the appropriate DNA-sequence recognition, DNA-binding, and RNA polymerase activity (Goodrich *et al.*, 1996). The second category of gene-regulating elements is classified as enhancers and is responsible for an increase in the rate of promoter-initiated transcription. A well-defined regulatory system, which is known to respond to either low or high iron levels, is called the IRE-IRP system (Haile, 1999).

The system involves IREs and IRPs that regulate expression on a transcriptional and post-transcriptional level (Pantopulos, 2004; Takahashi-Makise *et al.*, 2009). The presence of oxygen and/ or iron is required for the hydroxylation of the proline (Pro) residues in HIF-2α (hypoxia-inducible factor 2 alpha) by prolyl hydroxylase (PHD) (Takahashi-Makise *et al.*, 2009) (Figure 7 A). The von Hippel-Lindau tumour suppressor (pVHL- part of E3 ubiquitin ligase) subsequently ubiquitinates and degrades the hydroxylated HIF-2α, leading to suppressed levels of *DMT1* and *CYBRD1* transcription. HIF-2α is, however, not hydroxylated and degraded during periods of iron depletion. The factor is free to bind to HIF-1β (hypoxia-inducible factor 1 beta) and translocate into the nucleus to initiate the transcription of *DMT1* and *CYBRD1*. The translation of TRF1 and FTN mRNA is controlled by interactions between IREs, present at the 3' and 5' UTRs respectively, and IRPs (Papanikolaou & Pantopulos, 2005) (Figure 7 B). During periods of low iron concentration, IRP2 is free to bind to IREs upstream of storage proteins (ferritin) and downstream of iron uptake proteins (TfR1) to inhibit or enhance the translation of these mRNA sequences respectively (Takahashi *et al.*,

2009). In contrast, iron repletion signals the S-phase kinase-associated protein 1 (SKP1)-cullin-1 (CUL1)-F-box and leucine rich repeat protein 5 (FBXL5) E3 ubiquitin ligase complex (SCF) ubiquitination and degradation of IRP2. The translation of FTN is initiated while the rate of *TfR1* translation decreases.

IREs form loop and stem structures within UTRs and are evolutionary conserved among vertebrates, signifying the role of this system during eukaryotic development (Aziz & Munro, 1987). IRPs function as the iron sensors within the body and share high levels of homology and belong to a protein family consisting of Fe-S cluster isomerases. According to research on the IRP1 protein, it is the only one known to form 4Fe-S cluster type aconitase (catalyses isomerisation of citrate to isocitrate). The cluster determines the function and serves as a principle site of regulation. The cluster assembles in cells during periods of iron excess and remains inactive. For the IRP to bind to IREs, a gradual disassembly of the cluster must occur to allow IRP1 activation. This disassembly only occurs in iron-depleted cells. In addition, H_2O_2 and/ or nitrogen oxide (NO) can activate IRE-IRP binding and lead to elevated iron absorption *via* iron-bound-TfR1 complex engulfment. This activation (H_2O_2 specifically) can be antagonised by the presence of hypochoric acid, which is released during the myeloperoxidase reaction of the respiratory burst of certain phagocytic cells. IRP2 is synthesised in a *de novo* fashion during iron starvation and is actively degraded during conditions of high iron. IRE-containing mRNAs were identified by Johansson and Theil (2002), which encode for several proteins involved in energy and iron homeostasis not previously known to exhibit a defined IRE-IRP system. The research group identified FTN-like translation IREs within the 5' UTR of erythroid *ALAS2* and mitochondrial aconitase mRNA.

Several groups have categorised several different response elements within the *LTF* promoter (Zhang & Teng, 2000; Stokes *et al.*, 2004). An estrogen response element (ERE) was discovered within an estrogen response module localised to the *LTF* promoter. The ERE shares high levels of conservation between humans and mice, indicating its significant evolutionary role in iron metabolism.

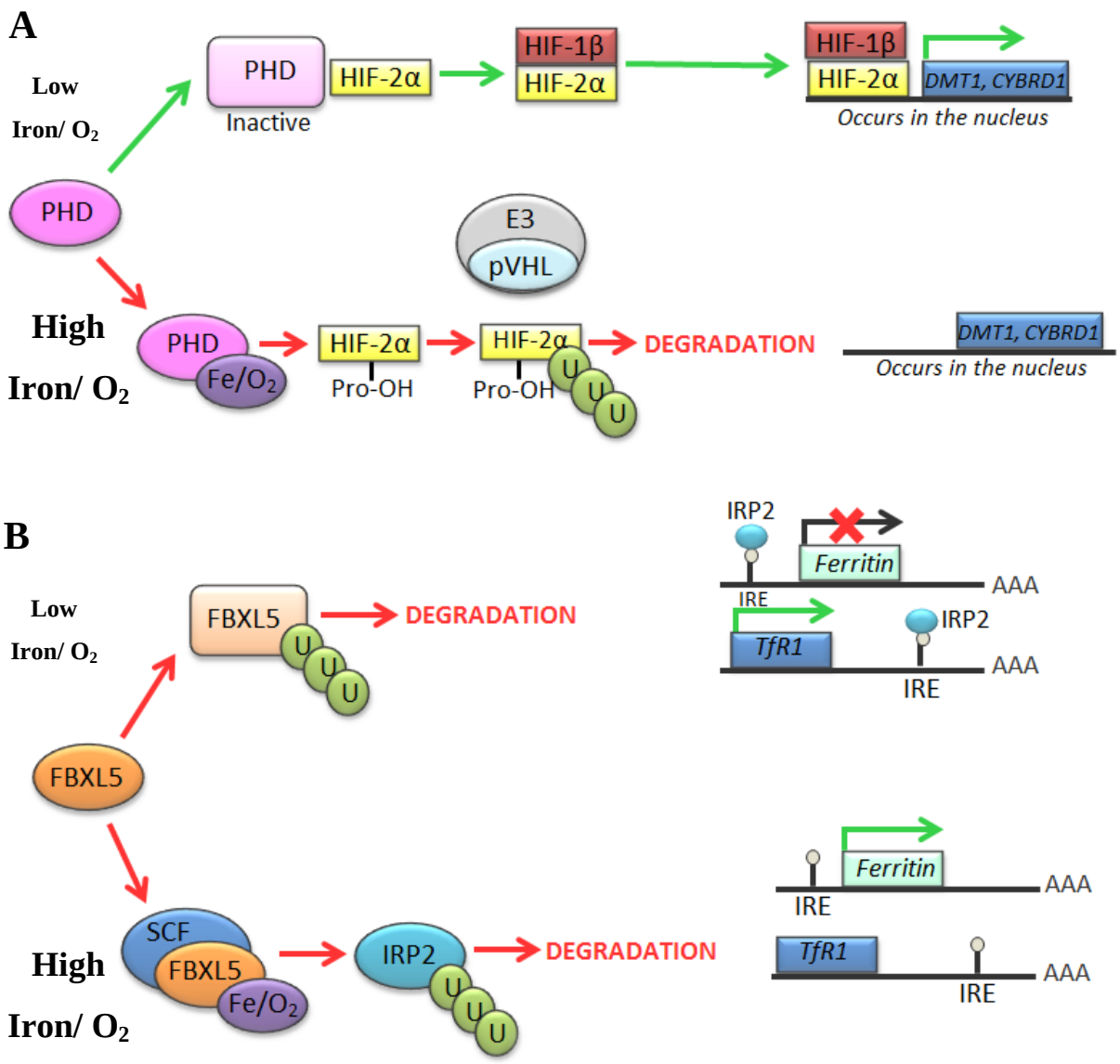

Figure 7 Transcriptional and post-transcriptional regulation of *DMT1, CYBRD1* and *FTN, TfR1* respectively

A= adenosine; *CYBRD1*= cytochrome b reductase 1; *DMT1*= divalent metal transporter 1; E3= ubiquitin ligase; FBXL5= F-box and leucine-rich repeat protein 5; H= hydrogen; HIF-2α= hypoxia-inducible factor 2 alpha; HIF-1β= hypoxia-inducible factor 1 beta; IRE= iron response element; IRP2= iron regulatory protein 2; O_2= oxygen; pVHL= von Hippel-Lindau tumour suppressor; PHD= prolyl-hydroxylase; Pro= proline; SCF= S-phase kinase-associated protein 1-cullin-1-F-box and leucine rich repeat protein 5 E3 ubiquitin ligase complex;*TfR1*= transferrin receptor 1; U= ubiquitin

A: Low oxygen and/ or iron result in HIF-2α stabilization which binds to HIF-1β and enhances *DMT1* & *CYBRD1* transcription. Iron surplus results in HIF-2α degradation and diminished *DMT1* & *CYBRD1* transcription. B: IRP2 is active during periods of iron depletion and translation of *FTH* and *FTL* is initiated. IRP2 is degraded during iron repletion and translation of *TfR1* commences.

In 1995, researchers demonstrated that the mouse version of the ERE also acted as a retinoic acid response element in two human cell lines (Lee *at al.*, 1995). This research proposes that

the estrogen response module controls the body's response to a multitude of hormones as it regulates the expression of *LTF*. Understanding the mechanisms employed to regulate iron aid in the explanation of how the differential expression of several genes or variable iron parameters could be linked to increased susceptibility to TB infection.

1.3 IRON AND TB INTERACTIONS

1.3.1 Genetic susceptibility to TB infection

The genetic makeup of the host affects the response initiated against pathogenic invasion (Russell *et al.,* 1997). The host's genetic profile may determine whether an infection will remain dormant or whether the pathogen will flourish. The differences noted between host responses to infection are controlled by the interaction between diseased macrophages and the host's immune system. Haldane's theory predicts that individuals with an African heritage are at a higher risk of contracting and becoming actively infected with TB when compared to those with a European heritage (Davies, 1995). The theory is based on the significantly vast spread of TB infection recorded after the Industrial Revolution in Europe. Disease resistance (immune response) was allowed to develop over a longer period in European individuals than when compared to African individuals. A study conducted on 2500 old-age home residents showed a two-fold increase in susceptibility to TB infection when black and white residents were compared (Stead *et al.,* 1990). Due to similar nutritional, physical, and social factors shared among residents, researchers concluded that the difference in susceptibility is caused by underlying genetic risk factors. Researchers have acknowledged that understanding the genetic changes associated with the difference between infection rates and outcomes among individuals are of great importance (Casanova & Abel, 2005). Association studies, utilizing certain candidate genes, produced significant evidence of HLA class II and solute carrier family 11 member 1 (*SLC11A1*) genes that correlated to increased susceptibility to TB infection (Alcais *et al.,* 2005). Gene expression profiling was performed using RNA, collected from TB inoculated mice, that was subsequently converted to cDNA for quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR) analysis (Beisiegel *et al.,* 2009). Researchers determined that several genes controlling different aspects of host immune response, chemokines, cytokines, and associated receptors, were differentially expressed in TB infected mice. Understanding the function, interactions, and expression of identified genes and protein products will play an important role in

increasing current knowledge of the pathogenesis of TB and those risk factors associated with elevated host susceptibility.

1.3.2 Iron overload and TB in African patients

The outcome of an autopsy-based study conducted in 1976 in a Johannesburg hospital showed that 18.3% of adults examined presented with elevated iron levels in the liver (MacPhail *et al.*, 1979). The recordings were six times higher than the reference range and were similar to recordings of European individuals suffering from *HFE*-linked hemochromatosis. The high frequency of iron overload in African individuals was originally attributed to the presence of elevated iron levels present in traditionally fermented beer that was consumed (Bothwell *et al.*, 1964). Traditional beer was fermented in iron drums or containers that would release high levels of iron into the beer. This environmental factor was deemed as the primary cause of reported iron overload in African populations until further investigation was initiated. Analogous results were obtained during a survey in Zimbabwe where 11.7% of 307 men tested were found to have significantly high serum-FTN levels and TSAT percentages. A striking 48.2% of adults in a Swaziland hospital presented with markedly high iron levels during liver biopsies. A survey conducted on the South African population expressed parallel outcomes in terms of hepatic iron concentrations (Gordeuk *et al*., 1996). Research emphasises that iron-overload remains a crucial health issue in Africa and the investigation into diet (iron intake), genes that regulate iron homeostasis, and its role in susceptibility to iron overload, is important. Due to a high frequency of iron overload and TB occurrence in African populations, researchers have focused their attentions on investigating whether or not a link exists between iron dysregulation and TB infection.

1.3.3 Experimental data provides insight into the iron and TB link

As early as 1978, researchers discovered that 36 of 71 Somali nomads suffering from iron deficiency exhibited a reoccurrence of suppressed infections after iron supplementation (Murray *et al.*, 1978). TB was among the infections that relapsed after the patient group and the control group received ferrous sulphate and aluminium hydroxide respectively. The research group provides two explanations for the results: the first is that the host organism actively deprives the invading pathogen of iron by increasing uptake and storage of free iron; the second explanation takes into account that the host's environment and food sources play a role in the availability of iron for proliferating pathogens. During iron depletion, the host cell generates a chalone-like substance that decreases DNA replication in an attempt to conserve

nutritional resources. The fact that the reduction in available iron decreases the rate of pathogen growth is auxiliary to the host's attempt to maintain reserves during periods of malnutrition. Regardless of the exact mechanism employed, researchers determined that iron deprivation suppresses infection while the opposite would increase the probability of infection relapse.

Lounis *et al.* (2001) set out to determine the effect iron treatment would have on the rate of proliferation of *M. tuberculosis* in Ba1b/C mice 6 weeks after being infected with the pathogen. Polymaltose ferric hydroxide was administered for 2 weeks prior to pathogen infection (6 weeks), and subsequently the mice were sacrificed to examine *M. tuberculosis* counts in the spleen and lungs. An equivalent number of mice received no additional iron but were also infected with the mycobacterium to be utilised as controls. The spleen weights were compared and the treated group showed higher values whereas overall body mass was not significantly different between the two groups. Colony forming units (CFU) counts of *M. tuberculosis* in the spleen and lungs were determined and a significant difference between the mice with normal and elevated iron levels was recorded. The difference in CFU counts indicates that elevated iron levels are linked to an increase in pathogen proliferation in the lungs and spleen of Ba1b/C mice. Microarrays were utilised by Wang *et al.* (2003) to determine the expression levels of a variety of genes with particular focus on the expression of genes that were altered during TB infection within the macrophage. Data obtained during this study demonstrates that the transcript levels of a number of key genes/proteins, including ribosomal proteins and chemokines, were altered during TB infection. Theurl *et al.* (2006) determined that inflammation caused and increase in iron retention in studied macrophages because iron could no longer exit the cell. Beisiegel *et al.* (2009) demonstrated that susceptibility to TB is dependent on the transcript levels of certain genes. More specifically, expression levels of genes that function within the innate immune response, iron transportation, and adaptive immune response were shown to be differentially expressed during infection. Previous studies have clearly demonstrated that differential expression of genes occur during TB infection. Therefore, the current study has developed from the need for further research endeavours which illustrate exactly which genes are affected, and to what extent they are affected, during infection with *M. tuberculosis.* Identified genes can subsequently be utilised as important markers for preventative screening, especially within developing countries.

During infection, interferon gamma (IFNγ) production increases since it facilitates the limitation of free iron that can be utilised by the pathogen to proliferate. DCYTB production is stimulated by the increase in IFNγ to make Fe^{2+} available to bind to NADPH oxidase, which is responsible for important antimicrobial purposes within the infected cell. This is expected because the increase in intracellular iron concentration within macrophages would result in an increase in iron available for the pathogen. Furthermore, unpublished work by Sanchez and colleagues demonstrated that DCYTB is indeed increased during microbial infection (Collins, 2008).

1.4 TUBERCULOSIS

Two primary types of the disease exist and are distinguishable from one another by the intended target region of infection. When the bacteria actively infect the lungs, the infection is referred to as PTB. When the infectious growth of *M. tuberculosis* occurs in other areas of the body unrelated to the pulmonary system, the infection is appropriately referred to as extrapulmonary TB. PTB is an infectious disease that is spread through the expulsion of the infectious agent, *M. tuberculosis*, from within the pulmonary system of an individual already infected with the disease (Daniel, 2005; Jeong & Lee, 2008). Active TB infection occurs at a higher frequency in men when compared to their female counterparts and primarily affects individuals between the ages of 15-59 years (Neyrolles & Quintana-Murci, 2009). This age group accounts for the most economically productive sector of a population and a high morbidity/mortality rate in this group directly affects the economic growth of a country. The importance of several awareness/prevention campaigns and the development of earlier detection methods are vital for continued population health, economic growth, and sustainability, especially in developing countries (Challu *et al.*, 2005; Gothankar, 2013).

1.4.1 *Mycobacterium Tuberculosis*-The causative agent

This devastating disease results from the active infection of an obligate human pathogen that was identified by Robert Koch in the 19th century and named *Mycobacterium tuberculosis* (Koch, 1882). Since obligate pathogens need to actively infect and subsequently cause disease in the host organism to survive, *M. tuberculosis* is aerosolised the moment an infected individual coughs and must be inhaled by another individual to ensure its own survival (Glickman & Jacobs, 2001). Once the invading pathogen enters the pulmonary system of its newly acquired host, bacilli infiltrate the host macrophages and continue to reside and proliferate within them. Due to iron limiting mechanisms employed by the host during

inflammation subsequent to *M. tuberculosis* infection, the pathogen experiences an environment of low iron concentration within the macrophage phagosome. *M. tuberculosis* has been under immense evolutionary pressure as it has continuously encountered an array of defensive strategies initiated by the host's immune system.

M. tuberculosis utilises Tf to sequester iron (Russell *et al.*, 1997). The pathogen then immediately employs several strategies to decrease iron storage and sequester iron from the host. Research utilizing mice as model organisms has shown that a minimum of 35 *M. tuberculosis* specific genes are induced or repressed as part of the pathogen's vital attempt to sequester iron to survive. Induced genes include those that encode for enzymes important for siderphore biosynthesis (*mtbA-J*) and repressed genes include *brfA* that encodes for a bacterial-specific FTN molecule responsible for iron storage (Dubnau *et al.*, 2005). Furthermore, *M. tuberculosis* releases several molecules called exochelins to actively sequester iron from the host stores and transport iron to mycobactin present on mycobacterium cell walls (Gobin & Howitz, 1996). Iron exochelin receptors (IERs) present on the cell wall bind to exochelins that have successfully sequestered iron from the host Tf carrier protein or the storage protein FTN (Brown & Ratledge, 1975; Hider & Kong, 2010) (Figure 8). An iron-siderophore reductase (ISR) subsequently reduces iron to enable entry of iron bound to Fxu cluster protein A, B, and C or mycobactin that cross the cell wall into the bacterial cytoplasm.

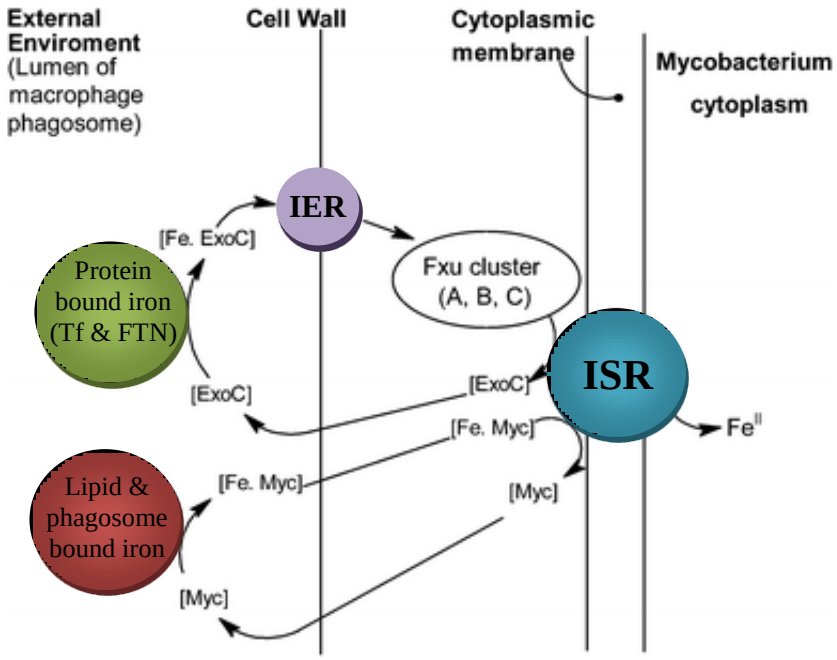

Figure 8 Both Mycobactin and Exochelin sequester iron from the host and deliver to the mycobacterial cytoplasm

ExoC= exochelin; FTN= ferritin; IER= iron exochelin receptor; ISR= iron siderophore reductase; Tf= transferrin

Adapted with permission from Hider and Kong (2010)

1.4.2 Immune response to *Mycobacterium tuberculosis*

A vast spectrum of microbes, which has been categorised as pathogenic invaders, illicit a specific immune response upon microbe exposure and/or inhalation (Houben *et al.*, 2006). *M. tuberculosis* gains access into host macrophages when bacilli bind to protein complexes present on macrophage cell membrane or through active phagocytosis. Phagocytosis is accomplished by signaling initiated by certain endotoxins that are released when bacteria are broken down (Ward *et al.*, 2011). A cholesterol-dependent mechanism involves the binding of the microbe to complement receptor type 3 (CR3) and thereby gains entry into the macrophage and is present in a phagosome. Microbial entry is thus initiated and completed without activating the anticipated immune response facilitated by macrophages (Gatfield & Pieters, 2000). During the immune response to infection, microbial phagosomes containing a microbe ligate to internal lysosomes for directed degradation of phagosome contents. *M. tuberculosis,* however, employs several proteins that prohibit the intended ligation, thereby bypassing the scheduled degradation process (Vergne *et al.*, 2004). Once mycobacteria are

phagocytised, infected macrophages move into certain tissues, which initiate the recruitment of further immune cells. The immune cells form a cluster referred to as a granuloma containing a central necrotic region in which bacteria, as well as neighbouring host cells, are destroyed. Resistant bacilli continue to survive within the macrophage, although they are present in a latent state and can be reactivated to cause disease at a later stage or under immuno-suppressive conditions (Grosset, 2003). IFN-γ plays a pivotal role in the immune system's response to mycobacterial invasion. A prime example involves an IFN-γ dependent guanosine triphosphatase (GTPase), referred to as LRG-47, that suppresses microbial proliferation in the absence of nitric oxide (NO) (MacMicking *et al.,* 2003). Researchers have not determined the exact mechanism of growth suppression induced by LRG-47; however, the protein regulates phagosome maturation, which is vital during host immune response to microbial invaders.

1.4.3 Epidemiology

As epidemiology is defined as the study of those elements that determine or influence the occurrence and spread of disease (in this case TB) in a specific population, this section will examine three principal elements, namely prevalence (number of existing cases), incidence (number of new or relapsing cases over a specific period), and the mortality rate (number of deaths directly resulting from the disease in a certain time period).

Several workshops were conducted on a worldwide scale to establish the estimated incidence, prevalence and mortality rates of TB in 2010 (WHO, 2010). It was estimated that 8.8 million incident cases were recorded and, of those recorded; a staggering 12%-14% were simultaneously living with human immunodeficiency virus (HIV). According to the World Health Organization (WHO) report for 2011, in terms of the prevalence of TB, South Africa (400 000 individuals) was ranked number 11 of 22 high TB burden countries (HBCs) for which the prevalence was recorded (WHO, 2011).

Alarmingly, third-world countries such as Mozambique, Ethiopia, Kenya, and Zimbabwe that have limited access to health care demonstrated a lower prevalence of TB in comparison to South Africa. South Africa does not exhibit the highest prevalence rate but, when comparing the amount of individuals that are infected with active TB to the population size, South Africa has the second highest percentage infected individuals of the 22 HBCs included in the 2011 WHO report. The report furthermore documented that an average of 25 000 South Africans

died from active TB infection (WHO, 2011). The overall outcome of the report emphasises the need of continued research into ways TB can be diagnosed with ease, less severe treatment strategies, and early identification of high risk individuals in South Africa. The WHO report for 2012 documented a 41% reduction in TB incidence in countries around the globe with the exception of those from Africa and Europe (WHO, 2012). The incidence rate of TB infection remains high in developing countries such as those in Africa (Figure 9).

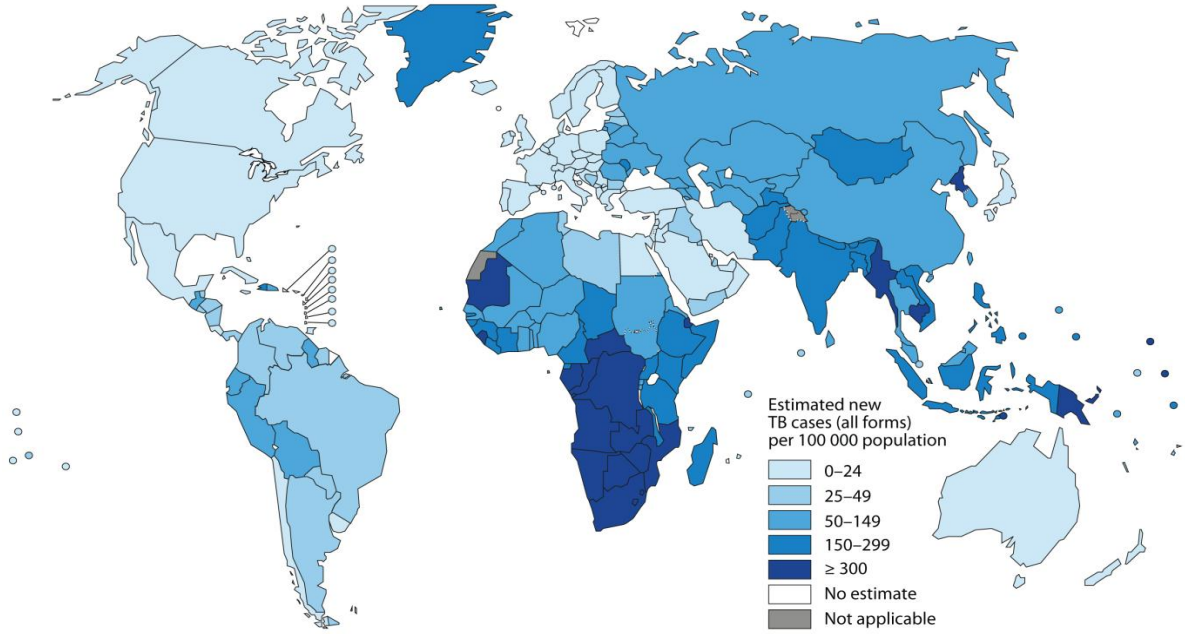

Figure 9 Estimated tuberculosis incidence rates for 2011 (WHO, 2012)

1.4.4 Diagnosis

Due to the non-specific symptoms related to TB infection, such as weight loss, fatigue, persistent coughing, loss of appetite, and night sweats, TB diagnosis is problematic (Jeong & Lee, 2008). For the physician to officially diagnose the patient with this infectious disease, several clinical, pathological, and radiological tests are needed to confirm active TB infection. The results obtained from the microscopic examination of exhaled sputum are also required to positively diagnose the patient with TB. Sputum samples are stained to test for the presence/absence of the acid-fast bacilli to aid in diagnosis. Cultures are then grown from the collected samples and once bacilli are visually detected, TB infection can be confirmed. A preliminary screen, referred to as the purified protein derivative tuberculin skin test, can initially be done in the physicians' office. A positive result supports a positive diagnosis; however, caution must be paid to a negative test result as not all negative results necessitate a

negative TB diagnosis. Due to the difficulty of the test and prolonged culture times required for the completion of TB screens, the physician will start the treatment regime, as the sooner the infection is treated, the better the prognosis (Donatin & Drancourt, 2012).

1.4.5 Treatment

The development of short-course chemotherapy, combined with directed observed treatment strategy (DOTS) and the Bacille Calmette-Guérin (BCG) vaccine, held great promise for the treatment and eventual eradication of TB worldwide (Wallgren, 1928). Although the treatment strategies are successful when the infected individual completes the course, research has shown that many do not follow the scheduled treatment, thereby rendering the treatment unsuccessful and *M. tuberculosis* drug resistance may develop (Surveillance, 1997). The primary aim of DOTS is to kill and eliminate bacilli, while the secondary aim is to prevent the development of drug resistance in the host. The strategy also centres on eradicating all stubborn bacilli to actively prevent the relapse of infection after treatment has been concluded (American Thoracic Society, CDC and Infectious Disease Society of America, 2003). Two primary drug types containing the vital bactericidal properties required to destroy invading pathogen have been developed. Rifamate® contains Isoniazid (INH) and rifampin (RIF), while Rifater® contains these two chemicals as well as Pyrazinamide (PZA). Research has shown that INH and RIF exhibit a potent killing ability that specifically targets *M. tuberculosis* that is actively multiplying throughout the first stage of therapy. The drug decreases *M. tuberculosis* infectiousness, thereby allowing the host to successfully combat the bacterium (Jindani *et al.*, 1980). PZA has the ability to actively eliminate bacilli that have become dormant within the host. This chemical is therefore of particular importance in the removal of persistent bacilli that may result in relapse (Girling, 1984).

Although TB is a curable disease, prevalence remains elevated due to poor adherence associated with long treatment periods and several severe drug-related side effects. Several research groups have contributed to the determination of the *M. tuberculosis* genome in recent years (Phillipp *et al.*, 1996; Cole *et al.*, 1998; Brosch *et al.*, 1998). These groups have acknowledged the importance of deciphering the genome to understand those genes and proteins that result in the high level of resistance and virulence. Drug therapies that halt the production of the complex lipid cell wall and other molecules that sequester nutrients from the host may aid in the eradication of this prevalent disease worldwide.

1.5 AIMS & OBJECTIVES

The aim of the current study is to experimentally measure the expression levels of several iron genes in patients that present with active TB and population-matched controls to establish whether significant correlations exist between iron parameters, gene expression, and susceptibility to developing active TB infection.

The first objective is to design primers to amplify pre-determined fragments of five genes (*CP, HAMP, HEPH, SLC11A1,* and *SLC11A2)* and optimise the conditions for optimal amplification of fragments for each gene. Accurate standard curves then need to be optimised for all 12 genes of interest (*CP, CYBRD1*, *FTH, FTL*, *HAMP, HEPH, HFE*, *HMOX1*, *LTF*, *SLC11A1, SLC11A2* and *SLC40A1*) as well as three reference genes (β-actin gene (*ACTB*), β-glucuronidase gene (*GUSB*) and ribosomal protein L37 gene (*RPL37A1*). Genes of interest were selected based on their primary role in actively affecting the rate of absorption, storage, export and transport in the blood stream. Blood samples then need to be collected from population-matched controls for comparative studies. The next objective is to extract RNA from the control group and reverse transcribed into cDNA for downstream RT-PCR. Expression levels of all 12 genes of interest need to be determined relative to reference genes. The final objective is to perform statistical analysis to determine whether significant correlations exist between iron parameters, gene expression, and TB susceptibility.

Chapter 2

DETAILED EXPERIMENTAL PROCEDURES

2. DETAILED EXPERIMENTAL PROCEDURES

2.1 PATIENT AND CONTROL GROUPS

Ethical approval was applied for and granted by the Human Research Ethical Review Committee from Stellenbosch University with specific reference number, N04/08/123. The patient group for the current study consists of 30 Xhosa-speaking, Black South-Africans that were diagnosed with active TB infection. The patient group age range (23-48years) and average (32.1± 1.34years) was comparable to the control group age range (29-47years) and average (33.67± 3.13years). The male to female ratio was 2:1 for the patient group while the control only consisted of male participants. Whole blood samples were previously collected from the Somerset West Clinic. Six randomly-selected population-matched controls were also included in this study. All the blood samples that were intended for downstream RNA extraction were collected in Ethylebediaminetetraacetic acid (EDTA) tubes while blood samples required for iron parameter screening were collected in BD Vacutainer® SST™ Serum Separation Tubes.

2.2 PARAMETER DATA

Iron parameters including serum-FTN, serum-iron, STF, and C-reactive protein (CRP) were measured according to standard procedures at two pathology laboratories. A panel of enzymes related to liver functionality and disease were also sent for testing. The parameters with corresponding normal references ranges in parentheses (where two values are present, the first represents the range demarcated by National Health Service Laboratory (NHLS) whereas the second is determined by Dr. Davies Pathologies are as follows: alanine aminotransferase (10-40U/l); albumin (30-48g/l); alkaline phosphatase (40-120U/l/ 40-150U/l); aspartate transaminase (8-20U/l/ 8-40U/l); C-reactive protein (0.0-10.00mg/l); conjugated bilirubin (0.00-8.6µmol/l); γ-glutamyltransferase (2-30U/l/ 0-65U/l); serum-FTN (15-322ng/mL); serum-iron (9-30µmol/L); STF (1.74-3.60g/L); total bilirubin (3.4-21µmol/L); TSAT (20-55%); total protein (60-85g/l) and unconjugated bilirubin (1.0-17.1µmol/L). Several descriptive statistics were generated for both groups which included the mean, median, standard deviation and 95% confidence intervals for each parameter under investigation (Table 2).

Table 2 Summarised iron parameters for patient and control groups

	TB group	Controls	TB group	Controls	TB group	Controls	TB group	Controls	TB group	Controls
Descriptive Statistic	S-Ferritin		S-Iron		S-Transferrin		TSAT (%)		S-Total Bilirubin	
Mean	219.00	86.00	14.76	16.35	2.32	2.37	26.72	27.67	11.43	5.50
Median	152.50	55.50	11.15	14.85	2.44	2.20	21.30	25.50	9.80	5.00
STD	222.64	74.07	13.08	3.98	0.50	0.43	22.39	6.77	7.17	0.84
Confidence Level(95.0%)	83.14	77.73	4.89	4.18	0.19	0.45	8.36	7.11	2.68	0.88

	S-Conjugated Bilirubin		S-Unconjugated Bilirubin		S-Total protein		S-Albumin	
Mean	4.78	2.50	7.04	3.00	80.55	74.67	37.90	43.83
Median	4.30	2.50	5.45	3.00	79.00	75.00	39.50	44.00
STD	2.26	0.55	4.88	0.63	8.91	2.94	6.72	1.72
Confidence Level(95.0%)	0.89	0.57	1.82	0.66	3.27	3.09	2.51	1.81

	S-Gamma Glutamyl Transferase		S-Alanine Aminotransferase		S-Aspartate Transaminase		S-Alkaline Phosphatase	
Mean	51.27	178.00	29.03	42.00	34.67	27.50	78.93	83.33
Median	30.00	50.00	22.50	31.50	30.00	24.00	74.50	74.50
STD	63.20	337.02	20.99	35.86	17.04	9.52	25.81	35.21
Confidence Level(95.0%)	23.60	353.68	7.84	37.63	6.36	9.99	9.64	36.95

Abbreviations: %= percentage; STD= standard deviation; TB= tuberculosis; TSAT= transferrin saturation

2.3 RNA EXTRACTION & PRECIPITATION

Previously, RNA was extracted for the patient group from whole blood and subsequently stored at -80^{o}C until further analysis. RNA extraction was performed using the QIAamp RNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's prescribed instructions. DNase-treatment was accomplished with either during RNA extraction *via* the on-column protocol using the specific RNase-Free DNase Set (Qiagen) or afterwards *via* the TURBO DNA-free^{TM} Kit (Ambion) both in line with manufacturers' protocols.

RNA integrity was confirmed by agarose gel electrophoresis and 260/280 ratios that were measured when samples were run on the Nanodrop ® ND-1000 spectrophotometer V3.0.1 (Nanodrop Technologies) (Table E1). An agarose gel (2% (w/v)) was made using 1X TBE buffer [90mM Tris ($C_4H_{11}NO_3$), 90mM Boric Acid (H_3BO_3), 1mM EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$) while 0.01% (v/v) ethidium bromide (EtBr) was used to stain the gel. Each RNA sample (10µl) with 1X RNA Loading Dye (Fermentas) was placed inside a 2720 Thermal Cycler (Applied Biosystems) for 10 minutes to heat the mixture to 70^{o}C. To confirm fragment length on the gel, 4µl of the RiboRuler^{TM} High Range RNA ladder (Fermentas) was

heated along with the samples to 70°C. Thereafter, all the samples as well as the ladder were kept on ice and loaded onto the agarose gel. The voltage was set at 120V and electrophoresis continued for 1 hour in 1X TBE buffer. The PCR products were thereafter visualised using ultraviolet light translumination on the Multigenius Bio Imaging System (Syngene).

The concentration of all RNA samples was ascertained with the Nanodrop® ND-1000 spectrophotometer V3.0.1 (Nanodrop Technologies) and samples were subsequently stored at -80°C once three volumes of 100% ethanol was added to each sample. Patient RNA was precipitated according to the following steps. Sodium acetate (0.3M) was added to each RNA sample and subsequently kept at -80°C for 20 minutes. Centrifugation at 12000 x g for 15 minutes followed and the supernatant was discarded. Thereafter, 500µl of ethanol (75%) was applied to the sample and then centrifuged at 12000 x g for 15 minutes. The supernatant was removed from each sample and the precipitated pellets air dried for several minutes. As the required end concentration of the RNA is 200ng/µl, differing volumes of RNase-free water provided in the Qiagen kit were calculated using initial RNA concentrations and were added to each sample to achieve the required end concentration.

2.4 FIRST-STRAND CDNA SYNTHESIS

The Go Script™ Reverse Transcription System (Promega) was utilised to convert RNA to cDNA *via* first-strand cDNA synthesis. cDNA synthesis was subsequently performed according to the instructions prescribed by the manufacturer. Oligo $(dt)_{15}$ (0.5ng) and random primers (0.5ng) were added to 500ng RNA (previously mentioned) to a final volume of 5µl. Reaction tubes were placed in a 2720 Thermal Cycler (Applied Biosystems) for 5 minutes at 70°C and then immediately placed on ice for an additional 5 minutes. Several reagents, including 1X Go Script™ reaction buffer, 2.5mM $MgCl_2$, 0.5mM PCR nucleotide mix, 20U recombinant RNasin ribonuclease inhibitor and 5% (v/v) Go Script™ reverse transcriptase were added to the previous reaction to a final volume of 20µl. This reaction was then incubated in a 2720 Thermal Cycler (Applied Biosystems) at 25°C for 5 minutes, at 42°C for 1 hour and 70°C for 15 minutes. cDNA samples were aliquoted and stored at -20°C.

2.5 GENE EXPRESSION ANALYSIS

2.5.1 Real-Time PCR

2.5.1.1 Oligonucleotide Primers

Primer pair sets were previously designed for *CYBRD1*, *FTH*, *FTL*, *GUSB*, *HFE*, *HMOX1*, *LTF* and *SLC40A1* and were available for use during this study. Oligonucleotide primers were designed for *CP*, *HAMP*, *HEPH*, *SLC11A1* and *SLC11A2* while previously published primers for *ACTB* and *RPL37A1* were identified and utilised in the study (Maeß *et al*., 2010). All the primer sets were designed in such a way that at least one of the two primers specifically covered an exon/intron boundary to ensure that contaminating gDNA was not amplified. Ensembl (www.ensembl.org) was utilised to gather all the reference sequences for the 8 genes under investigation (See Table 3 for accession numbers, primer design and other additional information) (Hubbard *et al.*, 2007). Primers were designed using the PrimerQuest (Integrated DNA Technologies) and LightCycler 480 software system (Roche Diagnostics).

2.5.1.2 Amplification of cDNA Sequences

Synthesised cDNA samples were amplified using the SYBR® Green I dye (Roche Diagnostics) and a Real-Time PCR Instrument. All experiments were performed on the Roche LightCycler 480 I or II (Roche Diagnostics) in white 96-well PCR microplates specific for the instrument (Figure 10). Each well contained 10µl reaction mix consisting of 1X LightCycler® 480 SYBR Green I Master (Roche Diagnostics), 0.4 or 0.5 pmol of each primer and 1µl of undiluted or 10X diluted cDNA sample (Table A1).

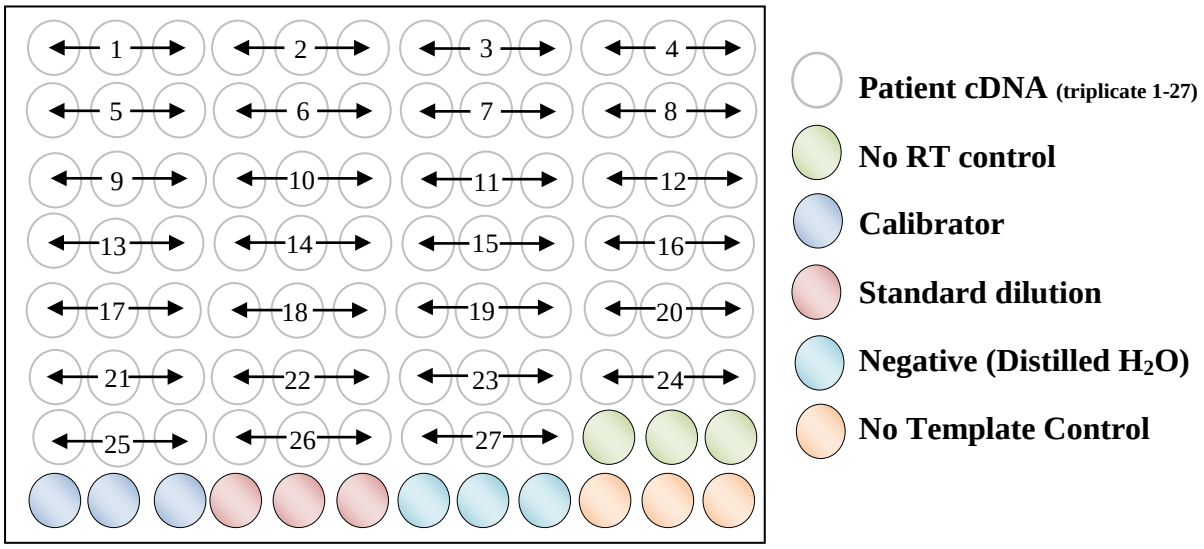

Figure 10 General format for LC480 96-well plate experimental setup

Table 3 Designed oligonucleotide primer sets and Real-Time PCR conditions for the amplification of synthesised cDNA

Gene Name	Ensembl Accession Number	Primer Name	Primer Sequence (5'- 3')	Primer volume (µl)		Product Size (bp)	T_M (°C)	T_A (°C)
				Version I	*Version II*			
**ACTB*	ENSG00000075624	ACTB F	ATTGCCGACAGGATGCAGAA	0.5	0.4	150	59.8	60/ 62
		ACTB R	GCTGATCCACATCTGCTGGAA	0.5	0.4		59.9	
CP	ENSG00000047457	CP F	AACATTACCAGGTGAAACTCTC	0.5	0.5	124	59.9	58
		CP R	GAGGTCCTTAACTTGATCCACA	0.5	0.5		59.9	
**CYBRD1*	ENSG00000071967	CYBRD1e1/2F	GGGCATCGCCATCATCGTCTACAG	0.5	0.5	133	61.3	65
		CYBRD1e2R	CTCAAACACGGCCACCACAGAG	0.5	0.5		60.2	
**FTH*	ENSG00000167996	FTH1 F	CCTGTCCATGTCTTACTACTTT	0.5	0.5	106	52.9	55
		FTH1 R	CAGTTTCTCAGCATGTTCCCT	0.5	0.5		57.5	
**FTL*	ENSG00000087086	FTL F	CCTACCTCTCTCTGGGCTTCT	0.5	0.4	122	57.3	55/ 58
		FTL R	TTGCATCTTCAGGAGACGCTC	0.5	0.4		59.9	
**GUSB*	ENSG00000169919	GUSB F	CTCATTTGGAATTTTGCCGATT	0.5	0.5	81	52.3	55
		GUSB R	CCGAGTGAAGATCCCCTTTT	0.5	0.5		54.4	
**HFE*	ENSG00000010704	HFEe5F	CGTCTGGCACCCTAGTCATTGGAG	0.5	0.5	111	60.8	65
		HFEe5/6R	CTCCTCTTGAACCCTGCCTCTTCC	0.5	0.5		60.7	
**HMOX1*	ENSG00000100292	HMOX1e2F	TTTGTCAGAGGCCCTGAAGGAGGC	0.5	0.5	119	62.7	65
		HMOX1e2/3R	CCATCACCAGCTTGAAGCCGTC	0.5	0.5		60.4	
**LTF*	ENSG00000012223	LCT F	GGGACCGAAAGACAGCCACGA	0.5	0.5	141	62.2	55
		LCT R	TATAGGGACATTCCATCCAGC	0.5	0.5		59.5	
**RPL37A1*	ENSG00000145592	RPL F	CGCCCAGAAGGTGACCAATGC	0.5	0.5	203	61.2	60
		RPL R	ATGCAACTGGAGGGAACTGGG	0.5	0.5		61.0	
**SLC40A1*	ENSG00000138449	SLC40A1e3/4F	CTTAAAGTGGCCCAGACCTCGC	0.5	0.5	132	59.9	65
		SLC40A1e4/5R	GCAGGAAGTGAGAACCCATCC	0.5	0.5		57.6	

*Primers previously designed. Abbreviations: 5'= 5-prime; 3'= 3-prime; °C= degrees Celsius; bp= base-pair; A= adenosine; *ACTB*= beta actin; C= cytosine; *CP*= ceruloplasmin; *CYBRD1*= cytochrome B reductase 1 gene; F= forward primer; *FTH*= ferritin heavy chain gene; *FTL*= ferritin light chain gene; G= guanosine; *GUSB*= beta-glucuronidase gene; *HFE*= haemochromatosis gene; *HMOX1*= haem oxygenase 1 gene; *LTF*= lactotransferrin gene; R= reverse primer; *RPL37A1*= ribosomal protein L37 gene; *SLC40A1*= solute carrier family 40 member 1 gene; T= thymidine ; T_A= annealing temperature; T_M= melting temperature

Certain settings were kept constant during experimentation and is described as follows: Initial pre-incubation step was set for 10 minutes at 95°C with a ramp speed of 4.4°C/s; Amplification began with a 10 second hold at 95°C at a ramp speed of 4.4°C/s and was followed by a 15 second hold at the pre-determined annealing temperature (T_A) provided in Table 4 for Version I and II with a ramp speed of 2.2°C/s; a 20 second hold at 72°C with a ramp speed of 4.4°C/s at which a single acquisition was taken. Melt curve took place with a 5 second hold at 95°C with a ramp speed of 4.4°C/s and was followed by a 1 minute hold at 45°C with a 2.2°C/s ramp speed. Continuous acquisitions were taken throughout at a rate of 5 acquisitions/°C at a ramp speed of 0.11°C/s. A cooling step was set with a 30 second hold at 40°C at a ramp speed of 2.2°C/s. Experimental cDNA for the genes for which the primers were previously designed were all run for 40 cycles. Experimental cDNA for the specific primer sets designed during this study were run under the same conditions with the exception that the run for *CP* continued for 50 cycles. Standard curves were set up for each gene under investigation with optimal efficiency values close to 2 and slope values close to 3.3 and a log concentration range of 6-2 (1x10E6 – 1x10E2) (Table 4 & Figure D2).

Table 4 Efficiency and slope values for gene expression standard curves

Gene	Efficiency		Slope	
	Version I	*Version II*	*Version I*	*Version I*
ACTB	1.99	1.97	-3.35	-3.39
CP	1.94	1.92	-3.48	-3.52
CYBRD1	2.02	1.97	-3.27	-3.40
FTH	1.96	1.91	-3.44	-3.56
FTL	1.99	1.86	-3.35	-3.70
GUSB	2.01	1.90	-3.29	-3.59
HFE	1.98	1.98	-3.36	-3.38
HMOX1	1.99	1.99	-3.35	-3.35
LTF	2.15	1.90	-3.01	-3.59
RPL37A1	1.96	1.98	-3.43	-3.37
SLC40A1	1.99	1.98	-3.35	-3.36

Standard curves were utilised to perform relative quantification in order to obtain quantification cycle (Cq) values for the respective runs. Melting curve analysis was performed to verify that only one fragment had been amplified and the expected band size of

the fragment of interest was verified on a 2% (w/v) agarose gel [1X TBE buffer [90mM Tris ($C_4H_{11}NO_3$), 90mM Boric Acid (H_3BO_3), 1mM EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$) and 0.01% (v/v) EtBr] that was electrophoresed for 1 hour at 120V in 1X TBE buffer. Each PCR product (5µl) as well as 5µl cresol red loading buffer [0.02% (w/v) cresol red, 0.34% (w/v) sucrose] were loaded in each well of the set agarose gel. A 2.5µl sample of the 100 bp O'GeneRuler (Fermentas) was added to one pre-selected well of each lane and electrophoresed alongside the PCR products. The agarose gel containing the PCR products was visualised using ultraviolet light translumination on the Multigenius Bio Imaging System (Syngene).

2.6 STATISTICAL ANALYSIS

2.6.1 Calibration and normalization

All gene expression data was obtained during triplicate runs performed three times on the Roche LC480 for each individual to obtain nine data points for each individual for the eight genes of interest and the three reference genes (Average values shown in Tables C1, C2 & C3). Where two rounds were performed (in the case of *CP*), only six data points were obtained. Relative quantification was performed using the standard curve method in which alterations in transcript expression is determine relative to the calibrator and subsequently normalised to reference gene expression levels. Concentration values were calibrated to the average of a single sample (referred to as the calibrator) present on each plate. This calibrated value was then normalised to the average of the reference gene calibrated concentrations for a specific individual (*please see calculation below for further clarification*):

Individual A (round 1): Gene 1 Conc. 1 Cal Conc. 1 Ref gene 1 Conc. 1 *(calibrated)*
Gene 1 Conc. 2 Cal Conc. 2 Ref gene 1 Conc. 2 *(calibrated)*
Gene 1 Conc. 3 Cal Conc. 3 Ref gene 1 Conc. 2 *(calibrated)*

$$\frac{\text{Gene 1 Conc. 1}}{\text{Average of Calibrator Conc. 1, 2 \& 3}} = X_1$$

$$\frac{X_1}{\text{Average of Ref gene 1 Conc. 1, 2 \&3 } \textit{(already calibrated)}} = Y_1$$

Y_1= Calibrated and normalised value (ratio) for the first concentration obtained for individual A. Repeated with Gene 1 Conc. 2 & 3 to obtained X_2, X_3 and Y_2, Y_3 to utilise for downstream statistical analysis.

The same analysis is performed for rounds two and three for each individual for each gene of interest and the corresponding reference genes with *CP* as an exception with only two rounds performed. Therefore Y_1-Y_9 was obtained for each individual for each gene and corresponding reference genes. Outlier determination was subsequently performed using Tukey's Rule during which the 1st and 3rd Quartile for the nine values was determined, subtracted from one another to obtain the Interquartile range (IQR). The lower and upper limits were then determined by subtracting the IQR x 1.5 from the 1st Quartile and adding the IQR x 1.5 to the 3rd Quartile. Those scores that fell below or above the lower and upper limits respectively were excluded from downstream statistical analysis. Once outliers were eliminated from the nine value-set, the remaining values for each patient (TB group) were averaged. Values identified by the IQR method were excluded from downstream statistical analysis if they correlated with recorded experimental. Kruskal-Wallis tests were utilised to determine if there are significant differences between the medians of the patient and control groups for the eight genes compared to the three reference genes.

2.6.2 Statistical analysis performed with the R software

Scatterplot matrices, Pearson correlation coefficients, Kruskal-Wallis tests, Fisher's exact tests, decision trees and principle component analysis (PCA) were performed using the R language and programming environment (R Development Core Team, 2011).

2.6.2.1 Scatterplot matrices and Pearson correlations

Correlations between the eight genes under investigation were tested with a significance level of 5%. Correlation testing was also performed between the four iron parameters and the other measured parameters separately. Scatterplot matrices demonstrated that all data sets were non-normally distributed and therefore non-parametric tests were utilised in this study.

2.6.2.2 Kruskal-Wallis test

The Kruskal-Wallis test was selected to test for significant differences in location of the study groups. The significance level was set at 5%.

2.6.2.3 Fisher's exact test

Contingency tables containing those study subjects that were classified as a control or patient were compared to those that were above or below the identified cut-off point for significantly

different gene expression patterns. Contingency tables were constructed to summarise the counts of patients and controls with gene expression levels above or below certain cut-off points for different gene expression patterns. Fisher's exact test was used to calculate the probability of the observed outcome under the null hypothesis of no association. The significance level was set at 5% and odds ratios were examined to quantify the association of having TB with a certain cut-off point (determined during decision tree analysis).

2.7 VALIDATION

2.7.1 Reference gene selection

GUSB plays a role in the production of bilirubin as it removes certain glucuronides from conjugated bilirubin (Kreamer *et al.*, 2001). ACTB plays a primary role in cellular microfilaments (Clarke & Spudich, 1977) and differential expression between control and TB patients is not anticipated. RPL37A1 is constitutively expressed within cells to maintain ribosomal complexes required for accurate translation during the cell cycle and differentiation and therefore acts as a suitable reference gene candidate in the current study (Shiawhwa & Bird, 1995). *GUSB* has previously shown low levels of variation when comparing gene expression profiles between controls and patients suffering from TB (Coomer, 2011). *ACTB* and *RPL37A1* were selected as a researcher group had previously optimised these genes as reference genes in THP-1 (monocyte) cells (Maeß *et al.*, 2010).

2.7.2 MIQE guideline satisfaction

Several specifications stated within the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were taken into account throughout the experimental design and execution to ensure accuracy and repeatability (Bustin *et al.*, 2009). This included the detailed description of experimental groups, collected samples, RNA extraction, and storage, instrumentation, cDNA conversion, and storage, qPCR target information, and primer design, protocol used during amplification, and melt curve analysis. Several rounds of optimization of target fragments and standard curves were completed and targets were confirmed on agarose gels (Figure D1). Samples were run in triplicate and three reference genes were employed to confirm reproducibility. A calibrator sample was utilised throughout the experimental execution to account for inter-plate variability and a description of statistical analysis is provided. Overall, several recommendations within the MIQE

guidelines were maintained throughout the experimental design and execution of protocols utilised in this study.

Chapter 3

RESULTS AND DISCUSSION

3. RESULTS & DISCUSSION

3.1 ABSTRACT

Data feedback from the WHO shows that an overall decrease of 41% in TB prevalence is demonstrated worldwide, with the exception of Africa and certain regions in Europe (lower reduction percentages). However, the mortality rate associated with TB remains high and research into effective treatments, increased education and identification of at-risk individuals is critical in the battle against the severe burden of TB. Understanding the factors that predispose certain individuals to TB infection (or relapse thereof) will aid in drug development and education on a global scale.

RT-qPCR was utilised to determine expression profiles for several iron genes (*CP, CYBRD1*, *FTH*, *FTL*, *HFE*, *HMOX1, LTF*, and *SLC40A1*) normalised to three reference genes (*ACTB, GUSB,* and *RPL37A1*). Our data show that *CYBRD1*, *HFE, HMOX1,* and *SLC40A1* displayed up-regulation in the TB group (n=30) compared to the control group (n=6). Additionally, conjugated, unconjugated, total bilirubin, and total protein show elevated levels in TB patients while albumin levels was significantly lower. *CP*, *FTH*, *FTL*, and *LTF* transcript levels remained similar between patient and control groups. Several iron, immunology and liver-function parameters were measured to determine iron status, infection state, and possible liver damage. TSAT and iron were positively correlated, while Tf and FTN levels were negatively correlated. Several other parameters show significant correlations, which may in future form part of a panel of parameter tests that are prescribed to determine TB susceptibility. Low iron levels were associated with decreased *CP/GUSB* levels and increased *HMOX1/GUSB,* levels while low TSATs were associated with increased *FTL/GUSB* and *SLC40A1/GUSB* levels and decreased *CP/GUSB.*

Elevated *CYBRD1* levels may predispose individuals to active TB infection as increased iron absorption may provide a constant supply of iron to the invading pathogen. Increased *HFE* and *SLC40A1* levels may predispose individuals to TB infection as iron is not taken by the Tf-TfR complex as it is inhibited by HFE and iron is more readily exported from duodenal cells by increased *SLC40A1* levels. *HMOX1* expression increase may result in increased erythrocyte recycling and the release of iron from haem molecules thereby providing the pathogen with an ideal iron reserve for continued proliferation. Gene and protein expression is often regulated at a post-transcriptional or post-translation level, respectively. The absence

of significant differences seen in the current study is a testament to these modifications. The quantification of iron-regulatory genes and proteins at several levels was required to fully understand the relationship between iron status, immune response, and TB susceptibility. Certain genes that display differential expression in this study signify the relevance of research in this field. Gene and downstream protein expression profiles aid in our understanding of the immune response towards TB and how differences in these expression levels could result in individuals demonstrating a higher risk of TB infection due to altered iron status and/or diminished immune response to infection.

3.2 INTRODUCTION

Iron is essential for both the infectious microbial pathogen (*M. tuberculosis*) and the host organism within which it proliferates. Consequently, an on-going competition ensues to obtain and utilise the iron present within the host organism. Upon infection, the host immediately counteracts the pathogen by reducing the available iron present in the system. *M. tuberculosis* was discovered in the 19th century by Robert Koch (Koch, 1882). An individual that suffers from TB infection will expel active *M. tuberculosis* bacilli when coughing which can then be inhaled by other individuals in the immediate environment. *M. tuberculosis* enters the macrophage through active phagocytosis or protein-protein interactions on the macrophage membrane. Macrophages stimulate surrounding immune cells to respond to pathogen invasion by the release of endotoxins during the degradation of *M. tuberculosis* (Ward *et al.*, 2011). The release of IFN-γ following microbe recognition aids the immune response to the foreign pathogen (MacMicking *et al.*, 2003).

More than a century has elapsed, yet the discovery and development of specific drug therapies that target this bacterial infection, especially multiple resistant strains, remain in their infancy (WHO, 2012). The Millennium Developmental Goal of reducing the incidence of TB infection by 50% by 2015 is well-underway with a 41% reduction reported in 2012. The report furthermore documents that Africa and several European areas did not show the same reduction in TB incidence. Although this report is positive, the burden of TB remains severe (1.4 million TB-related deaths) and strategies to identify at-risk individuals and reduce this risk with education and predictive testing are of critical importance. Predictive testing could involve the identification and establishment of gene profiles that may predispose certain individuals to infection when environmental factors are similar among individuals.

A plethora of iron regulating genes has been identified and the expression/function of these genes and encoding proteins have become the focus of multiple research groups. The convoluted journey of iron begins with a reduction reaction controlled by DCYTB (Riedel *et al.,* 1995; Oakhill *et al.,* 2008). Iron is either stored as part of FTN in the labile store or it is exported into the blood stream through FPN on the baso-lateral membrane of duodenal enterocytes (Harrison & Arosio, 1996; Beaumont, 2010). Fe^{2+} is oxidised for to be iron exported and transported in the bloodstream by HEPH and CP respectively (Neifakh *et al.,* 1969; Han & Kim, 2007). Iron subsequently binds to Tf or LTF to facilitate distribution throughout the body (Sorensen & Sorensen, 1939; Yang *et al.,* 1984). HFE competes for iron-bound Tf on the TfRs on cell membranes during periods of high intracellular iron to prevent the accumulation of excess iron in tissues (Feder *et al.,* 1996). HMOX1 is induced during periods of high intracellular haem concentrations to break haem down and release cyto-protective by-products: CO and biliverdin (Kutty *et al.,* 1994).

Several lines of evidence have been published that describe the intricate relationship that exists between iron status, immune response and TB susceptibility (Murray *et al.,* 1978; Lounis *et al.,* 2001; Wang *et al.* 2003; Theurl *et al.,* 2006). These results demonstrate the need for further exploratory research into the exact mechanisms that regulate this relationship and whether aberrations in this relationship could explain an individual's susceptibility to TB.

3.3 SUMMARISED METHODS & MATERIALS

The reader is referred to Chapter 2 for an in-depth explanation of methods employed during this research project.

3.4 RESULTS

3.4.1. Increased expression of *CYBRD1*- a key role player in iron absorption

A differential increase in gene expression was recorded for TB patients when normalised to *GUSB* ($p<0.005$), *ACTB* ($p<0.05$), and *RPL37A1* ($p<0.01$). More specifically, a 2.23-fold increase was demonstrated between control individuals (1.08 ± 0.08) and TB patients (2.41 ± 0.33) when compared to *GUSB* expression (Figure 11 A). A 1.99-fold increase was reported when normalised to *ACTB*, with an average for controls (1.09 ± 0.18) and TB patients (2.18 ±

0.15) (Figure 11 B). When normalised to *RPL37A1*, a 3.26-fold increase in TB patients (3.76 ± 0.29) was seen compared to controls (1.15 ± 0.25) (Figure 11 C).

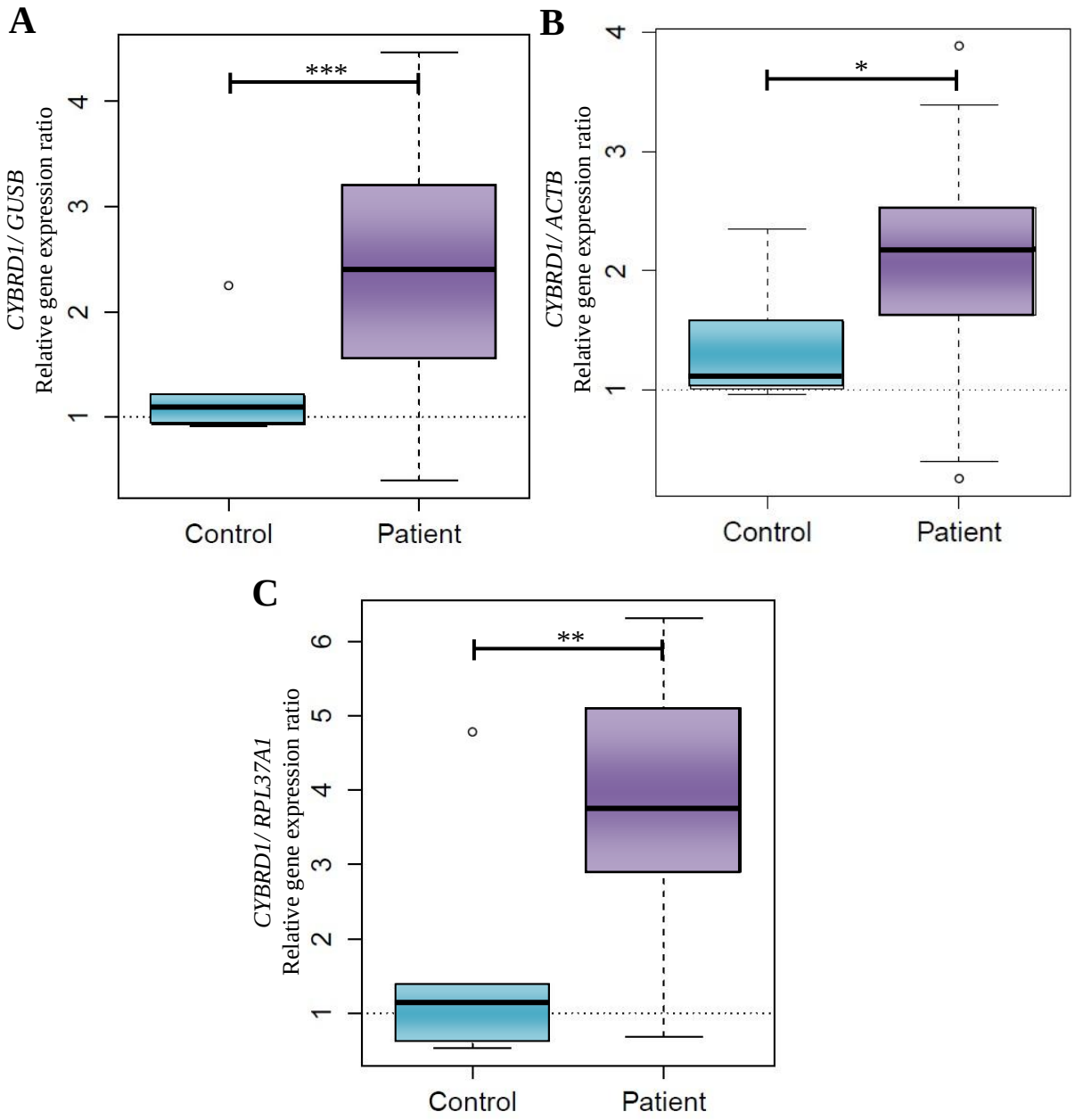

Figure 11 Increased *CYBRD1* expression measured between control and patient groups

A: Box plot representing the gene expression ratios of control vs. TB patients for *CYBRD1* normalised to *GUSB* ($^{***}p<0.005$). **B:** Box plot representing the gene expression ratios of control vs. TB patients for *CYBRD1* normalised to *ACTB* ($^{*}p<0.05$). **C:** Box plot representing the gene expression ratios of control vs. TB patients for *CYBRD1* normalised to *RPL37A1* ($^{**}p<0.01$).

3.4.2 Unaffected expression demonstrated for both iron-storage genes

The expression levels of the light- and heavy-chain proteins responsible for iron storage throughout the body were evaluated. No significant differences between levels measured in

control and patient individuals for both subunits for *GUSB* and *ACTB* were observed (Figures 12 & 13). Significant differential expression patterns for *FTH* and *FTL* normalised to *RPL37A1* is shown ($p<0.05$) (Figures 12 C & 13 C). 1.90-fold and 1.49-fold changes were documented for *FTH* (control: 0.96 ± 0.13; patient: 1.82 ± 0.17) and *FTL* (control: 1.00 ± 0.09; patient: 1.50 ± 0.22) expression for TB patients when normalised to the third reference gene (*RPL37A1*).

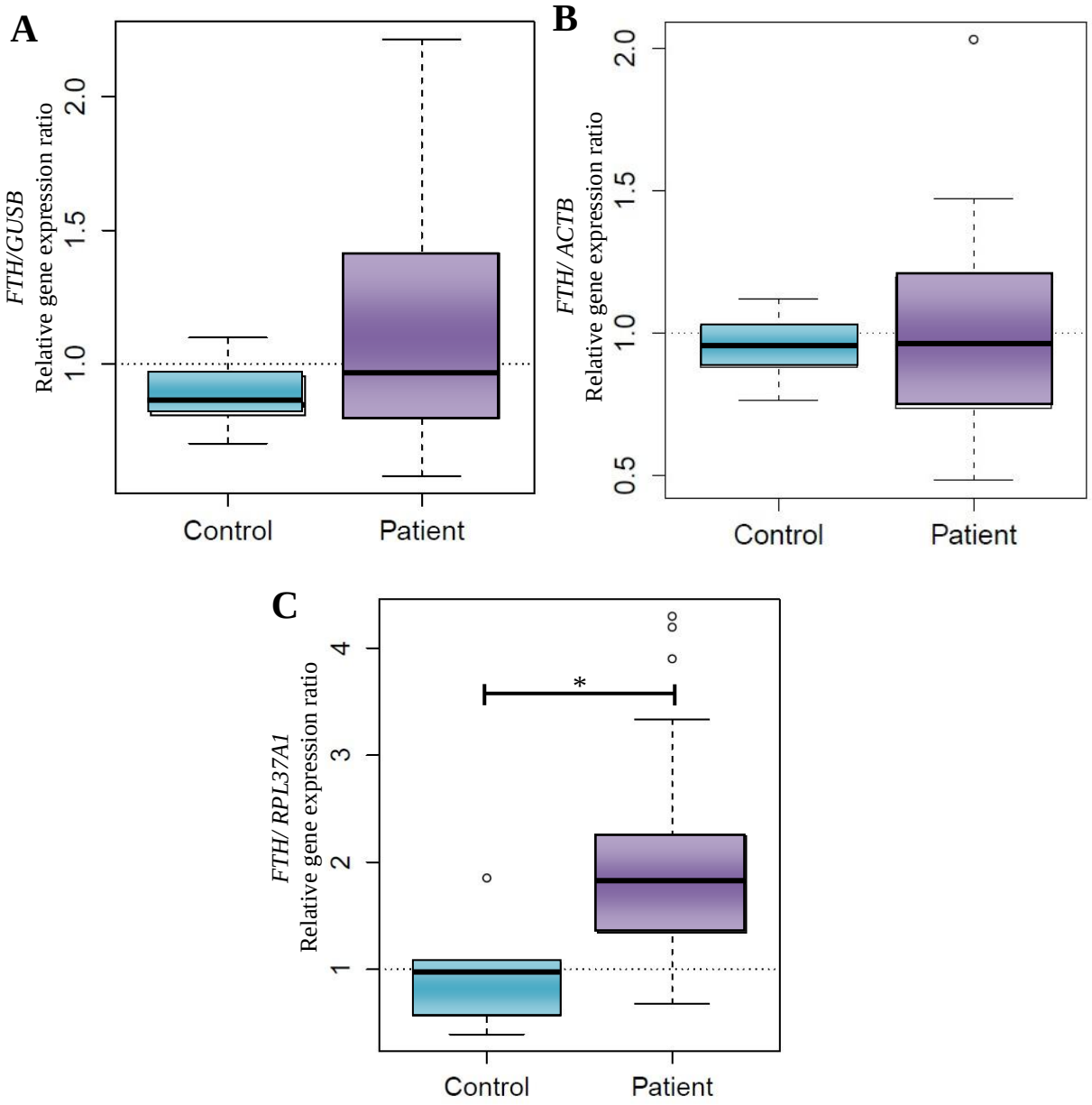

Figure 12 *FTH* expression remains unaltered between study groups

A: Box plot representing the gene expression ratios of *FTH* normalised to *GUSB*. **B:** Box plot representing the gene expression ratios of *FTH* normalised to *ACTB*. **C:** Box plot representing the gene expression ratios of *FTH* normalised to *RPL37A1*.

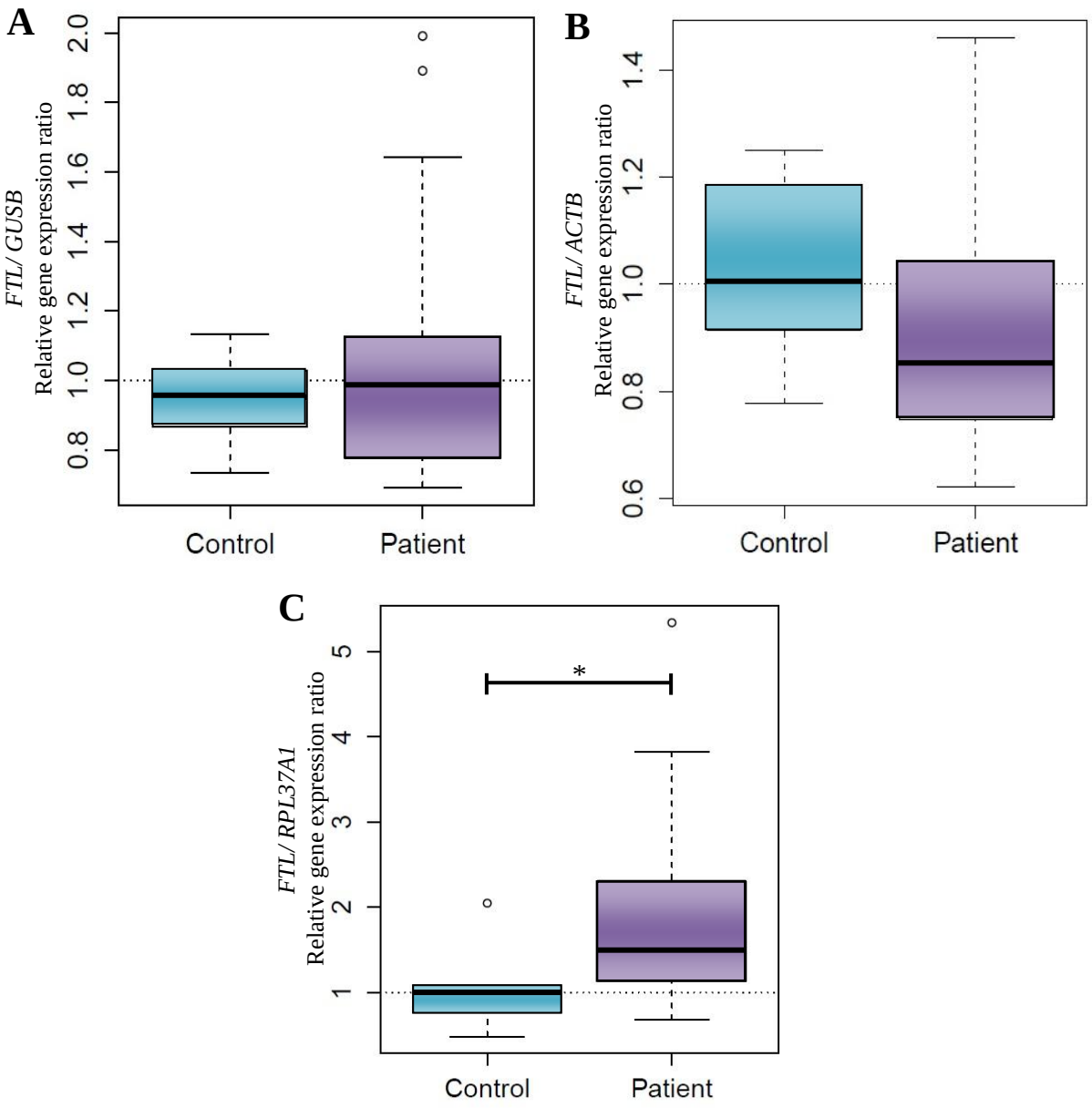

Figure 13 *FTL* expression shows no significant differential expression when the patient group was compared to the population-matched control group

A: Box plot representing the gene expression ratios of *FTL* normalised to *GUSB*. B: Box plot representing the gene expression ratios of *FTL* normalised to *ACTB*. C: Box plot representing the gene expression ratios of *FTL* normalised to *RPL37A1*.

3.4.3 Plasma ferroxidase remains unaffected in TB patients

The ferroxidase gene important in iron export showed an insignificant down-regulation for *GUSB* and *ACTB* between healthy control individuals (1.02 ± 0.03 and 1.03 ± 0.21) and TB patients (0.85 ± 0.08 and 0.94 ± 0.20) (Figure 14 A & B).

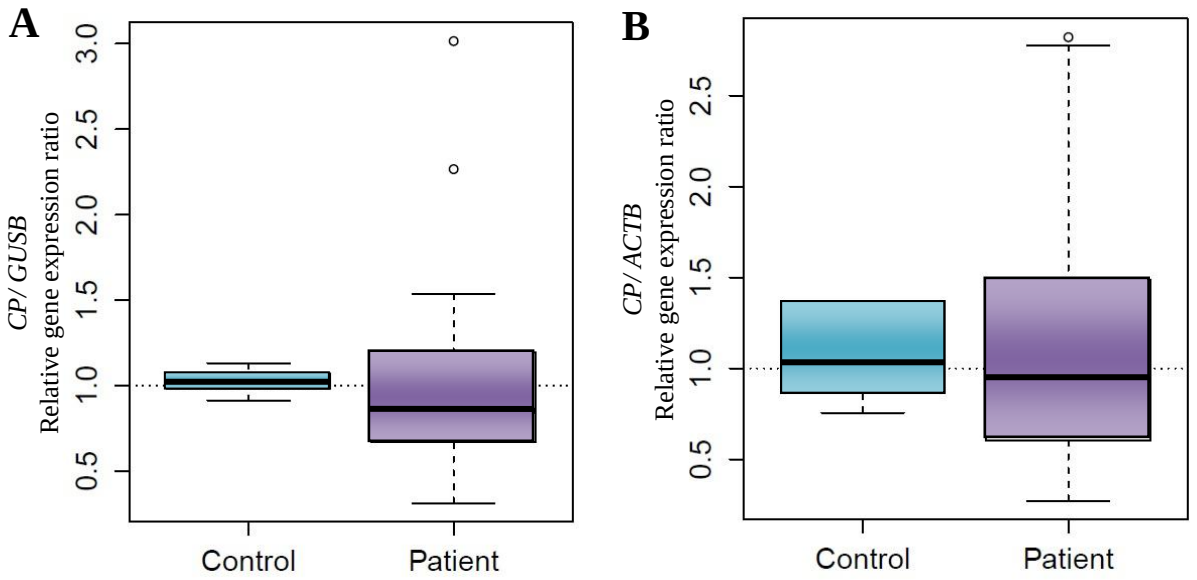

Figure 14 No significant difference in *CP* mRNA expression was observed when control individuals were compared to TB patients

A: Box plot representing the gene expression ratios of *CP* normalised to *GUSB*. B: Box plot representing the gene expression ratios of *CP* normalised to *ACTB*.

3.4.4 *LTF* expression profiles remain unchanged in patients while *SLC40A1* is elevated

The expression of *LTF* remained unaffected when control individuals where compared to TB patients for *GUSB* (0.60 ± 0.14 and 0.57 ± 0.16), *ACTB* (0.69 ± 0.11 and 0.62 ± 0.14) and *RPL37A1* (0.91 ± 0.47 and 0.80 ± 0.26) (Figure 15 A, B & C).

SLC40A1 expression was significantly up-regulated in TB patients in comparison to controls ($p<0.01$ when normalised to *ACTB* and $p<0.005$ when normalised to *GUSB* and *RPL37A1*) (Figure 16). The most significant increase between the control group (1.29 ± 0.22) and TB group (5.25 ± 0.36) was demonstrated for *RPL37A1* relative ratios (Figure 16 C). When normalised to *GUSB* and *ACTB*, control individuals (1.51 ± 0.32 and 1.61 ± 0.23) and TB patients (2.78 ± 0.30 and 2.51 ± 0.22) also illustrated significant increases in *SLC40A1* gene expression ratios (Figure 16 A & B). A 4.08-fold change was documented for *RPL37A1* normalization with lower changes seen for *GUSB* (1.84) and *ACTB* (1.56).

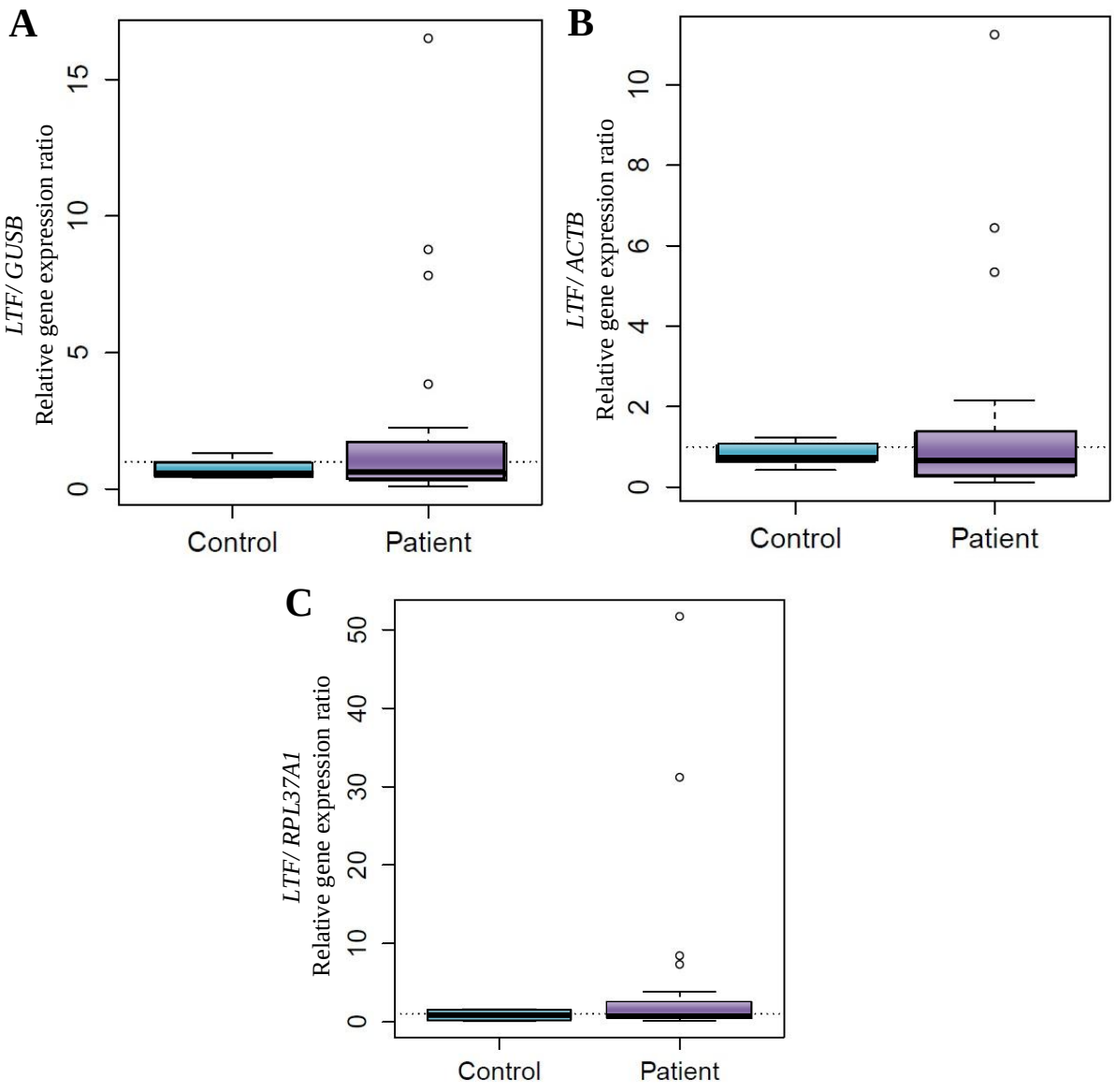

Figure 15 No differential *LTF* expression was demonstrated between the control and patient groups

A: Box plot representing the gene expression ratios of *FTL* normalised to *GUSB*. B: Box plot representing the gene expression ratios of *FTL* normalised to *ACTB*. C: Box plot representing the gene expression ratios of *FTL* normalised to *RPL37A1*.

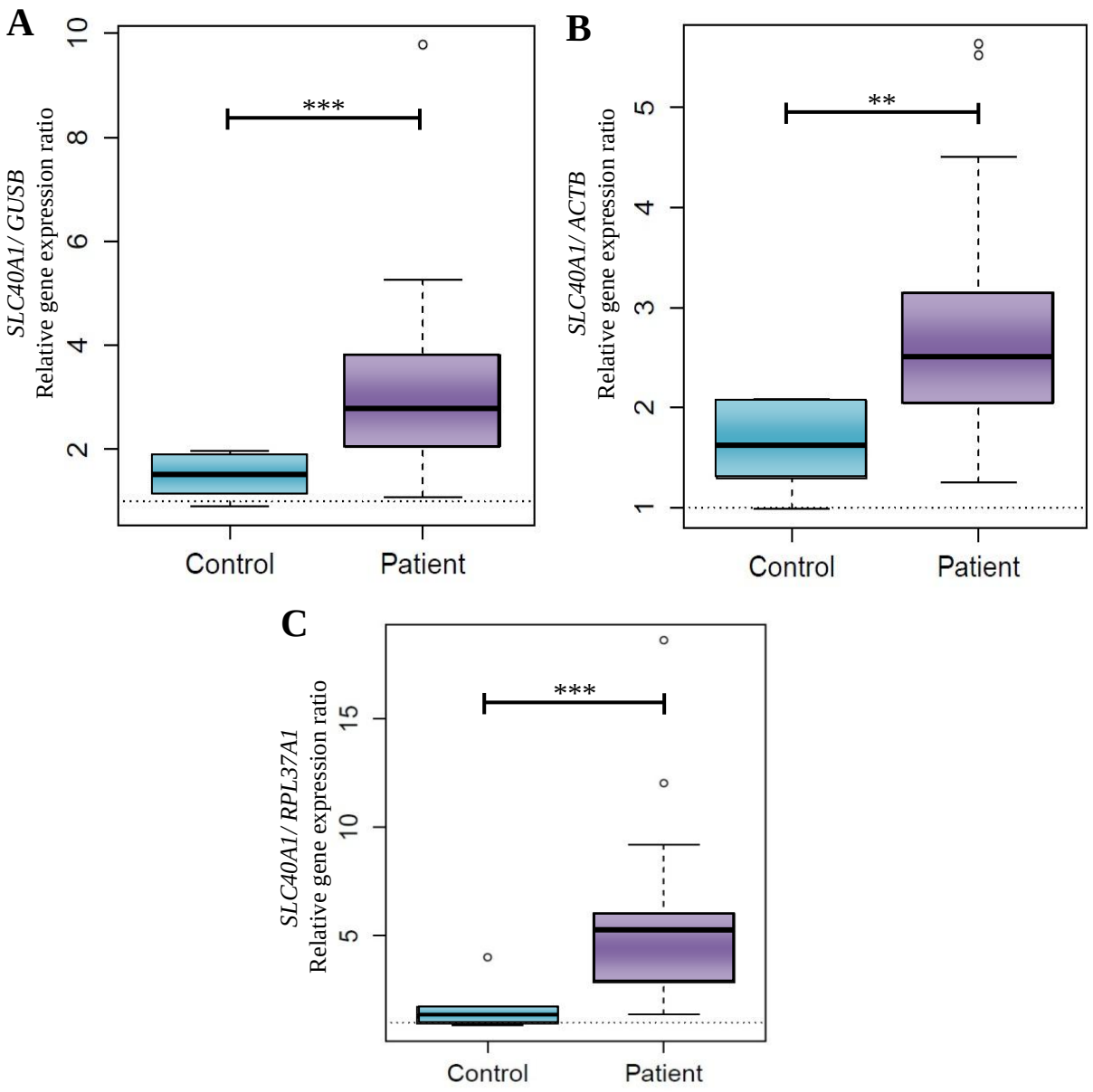

Figure 16 *SLC40A1* gene expression was up-regulated when study groups were compared

A: Box plot demonstrates the significant increase in *SLC40A1* (normalised to *GUSB*) expression ratios in TB patients vs. control individuals ($^{***}p<0.005$). B: Box plot demonstrating the significant increase in gene expression ratios of TB patients vs. control individuals for *SLC40A1* normalised to *ACTB* ($^{**}p<0.01$). C: Box plot demonstrating a significant increase in *SLC40A1* gene expression ratios of TB patients compared to controls when *RPL37A1* was utilised as the reference gene ($^{***}p<0.005$).

3.4.5 *HFE* gene expression is elevated in TB patients

The transcript levels of *HFE* were shown to be up-regulated in the patient group when ratios were normalised to all three reference genes employed during the study. An increase in gene expression was documented for TB patients when normalised to *GUSB* ($p<0.005$), *ACTB* ($p<0.05$), and *RPL37A1* ($p<0.005$) (Figure 17 A, B & C).

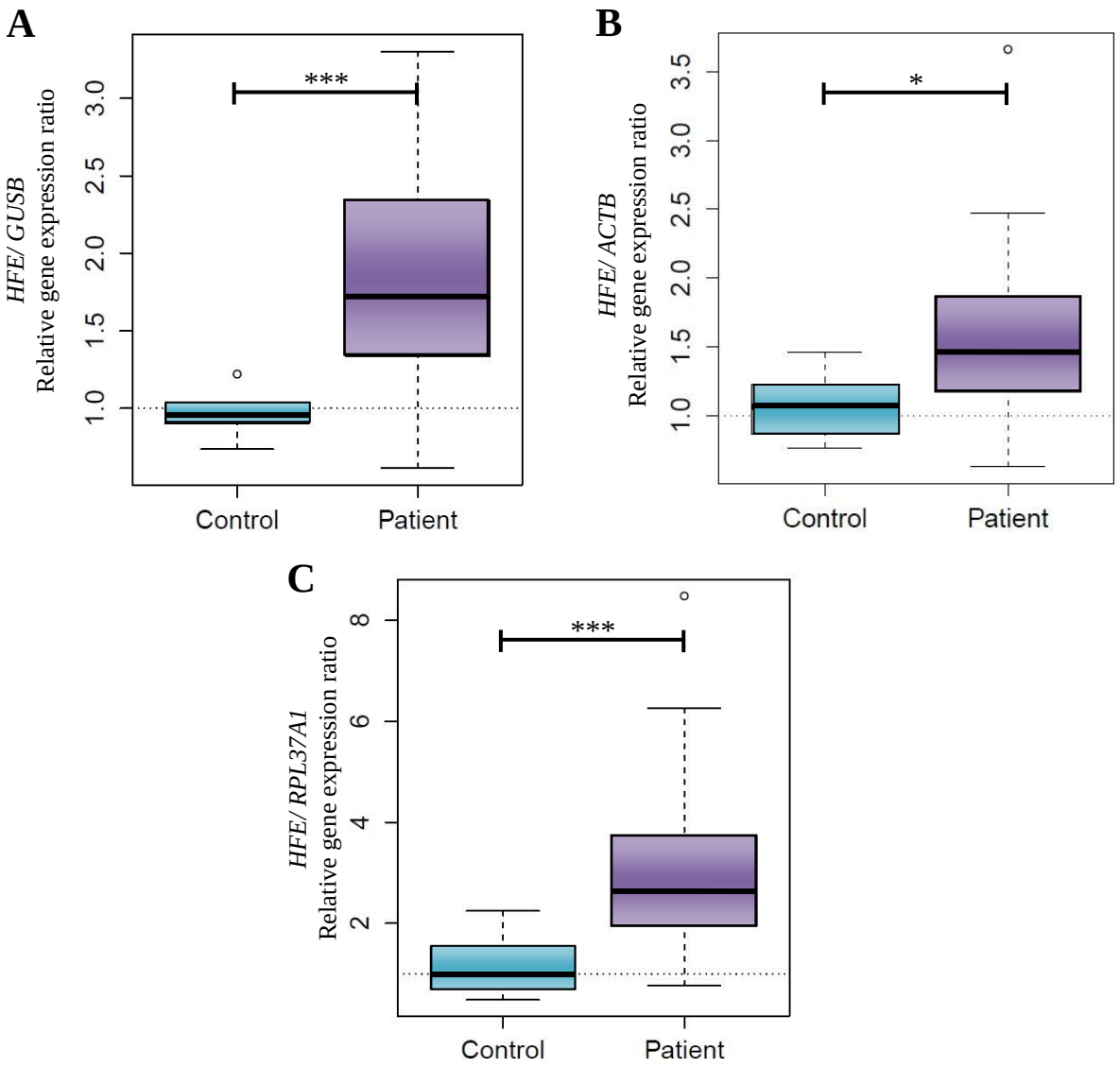

Figure 17 A significant increase in *HFE* gene expression was demonstrated in the TB patient group

A: Box plot shows that the *HFE* (normalised to *GUSB*) expression ratio is significantly increased in TB patients in comparison to control individuals ($^{***}p<0.005$). B: Box plot shows that the gene expression ratio of *HFE* normalised to *ACTB* is significantly increased in the TB group vs. control group ($^{*}p<0.01$). C: Box plot shows that the *HFE* gene expression ratio when normalised to *RPL37A1* was significantly elevated when patient and control groups were compared ($^{***}p<0.005$).

A significant 1.81-fold, 1.37-fold and 2.68-fold change was demonstrated between control individuals and TB patients for *GUSB*, *ACTB*, and *RPL37A1*, respectively. Displaying the most significant attenuation was *RPL37A1*, with a recorded control group median of 0.97 ± 0.26 and patient group median of 2.61 ± 0.36.

3.4.6 Key role player in iron-recycling (*HMOX1*) is up-regulated

HMOX1 transcript levels was quantified and *GUSB* ($p<0.005$) and *RPL37A1* ($p<0.005$) normalised ratios were significantly increased between control (1.28 ± 0.15 and 1.21 ± 0.25) and patient groups (2.05 ± 0.10 and 2.93 ± 0.24). Figure 18 A illustrates the up-regulation of *HMOX1* in TB patients with a computed 1.60-fold change while figure 18 C illustrates the up-regulation of this gene in the patient group with a 2.42-fold change.

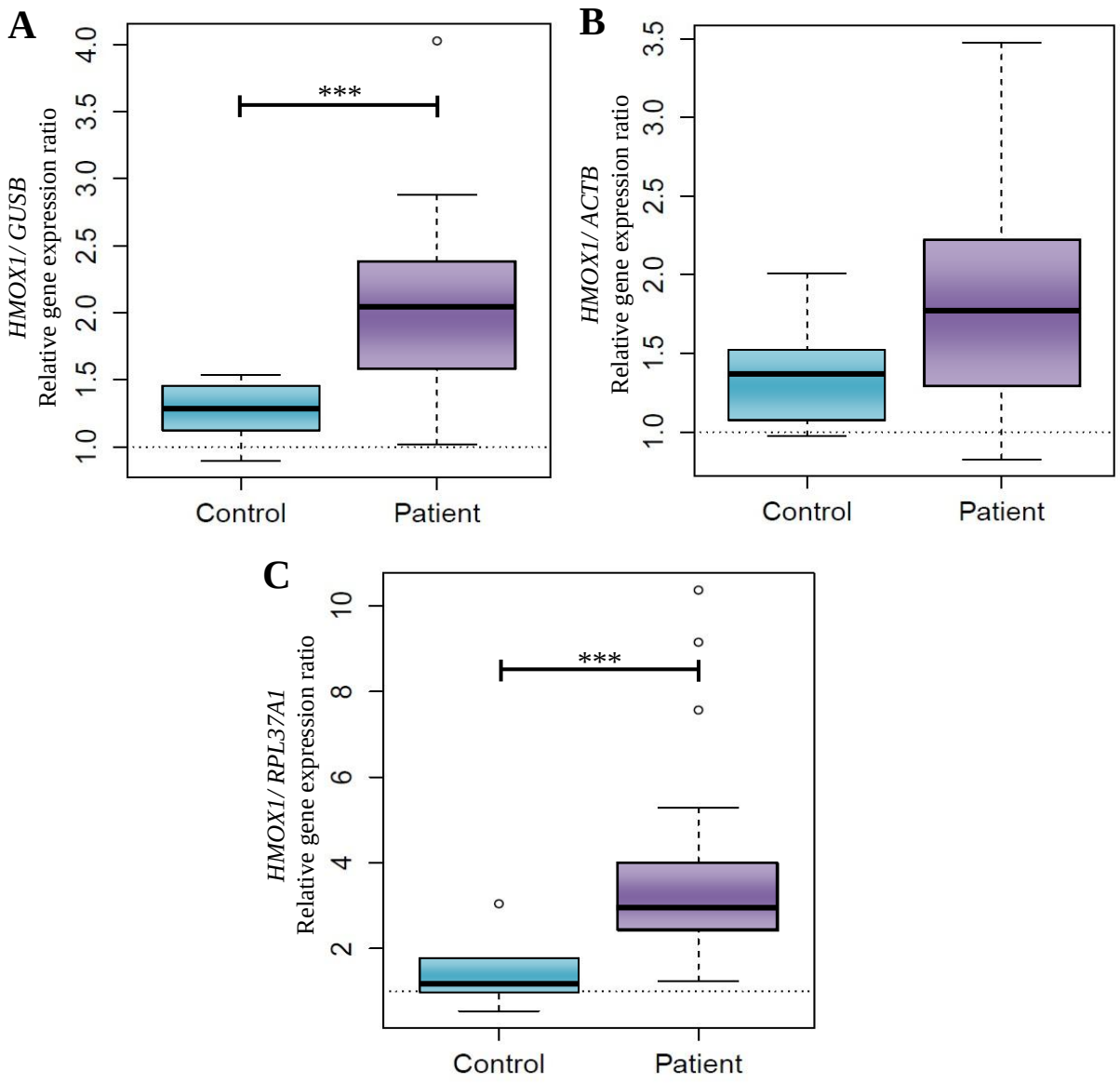

Figure 18 Significantly elevated levels of *HMOX1* was documented in the TB patient group compared to the controls

A: Box plot illustrating a significant increase in *HMOX1* (normalised to *GUSB*) expression ratio in the TB group when compared to the control group ($^{***}p<0.005$). B: Box plot illustrates a non-significant increase in *HMOX1* expression when normalised to *ACTB*. C: Box plot illustrating a significant increase in the *HMOX1* gene expression ratio in TB patients when *RPL37A1* was utilised as the reference gene ($^{***}p<0.005$).

3.4.7 Decision tree outputs

Optimal cut-off points for genes demonstrating significant differences between study groups were determined by decision tree analysis. For *HFE* expression ratios, normalised to *GUSB* a significant expression ratio cut-off point of 1.2 was found in the study cohort (Figure 19). The first split into nodes 2 & 3 indicates that 100% of 25 individuals that displayed a relative ratio greater than 1.2 were TB patients (n=25). Node 2 demonstrates that 55% of 11 individuals were controls (n=6) and that they displayed a ratio less than 1.2. Fisher's exact test demonstrated that a cut-off value of 1.2 is significant ($p<0.05$) and an odds ratio of 21.93 was recorded.

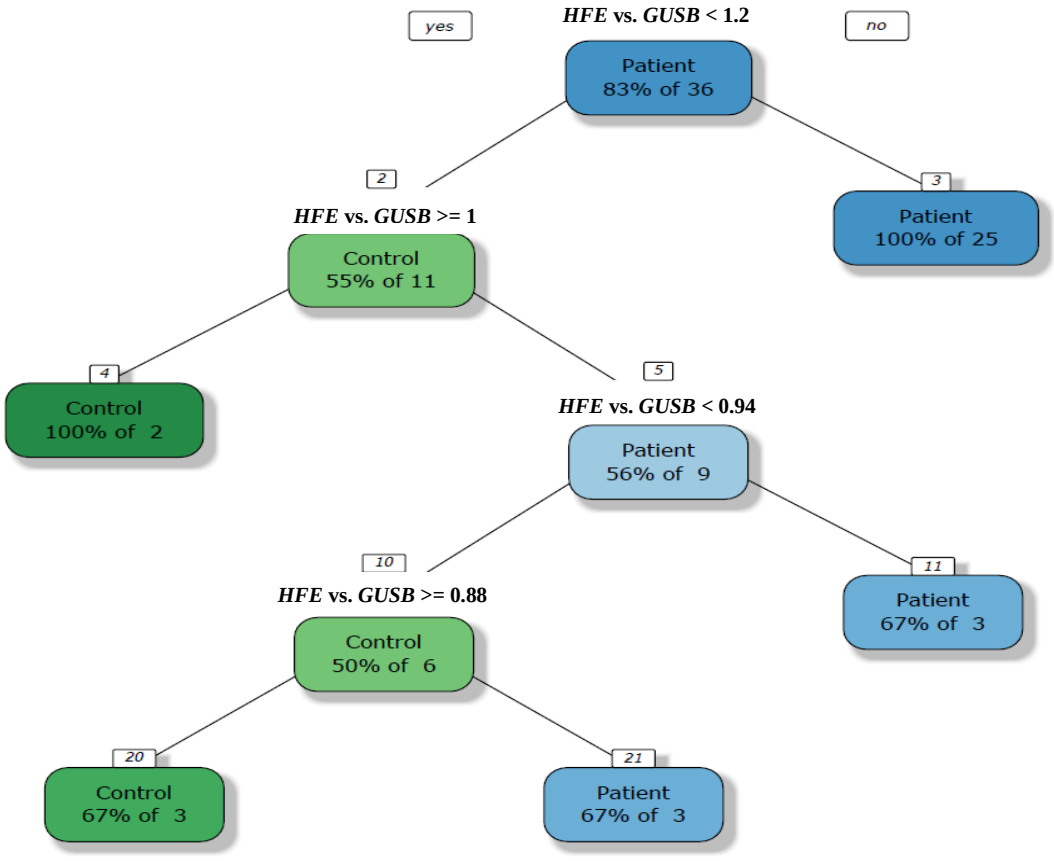

Figure 19 Decision tree showing the relative ratio cut-off point of 1.2 for *HFE/GUSB*

Decision tree analysis yielded a significant ($p<0.05$) cut-off point of 1.2 for expression ratios of *CYBRD1* normalised to both *GUSB* (Figure 20) and *ACTB* (Figure 21). In node 3 of *CYBRD1/GUSB*, 97% of 29 individuals were TB patients (n=28) and displayed a relative ratio greater than 1.2, while 71% of 7 individuals were controls (n=5) and displayed a ratio less than 1.2. Fisher's exact test demonstrated a 52.9 and 9.13 odds ratio for *CYBRD1/GUSB* and *CYBRD1/ACTB* respectively.

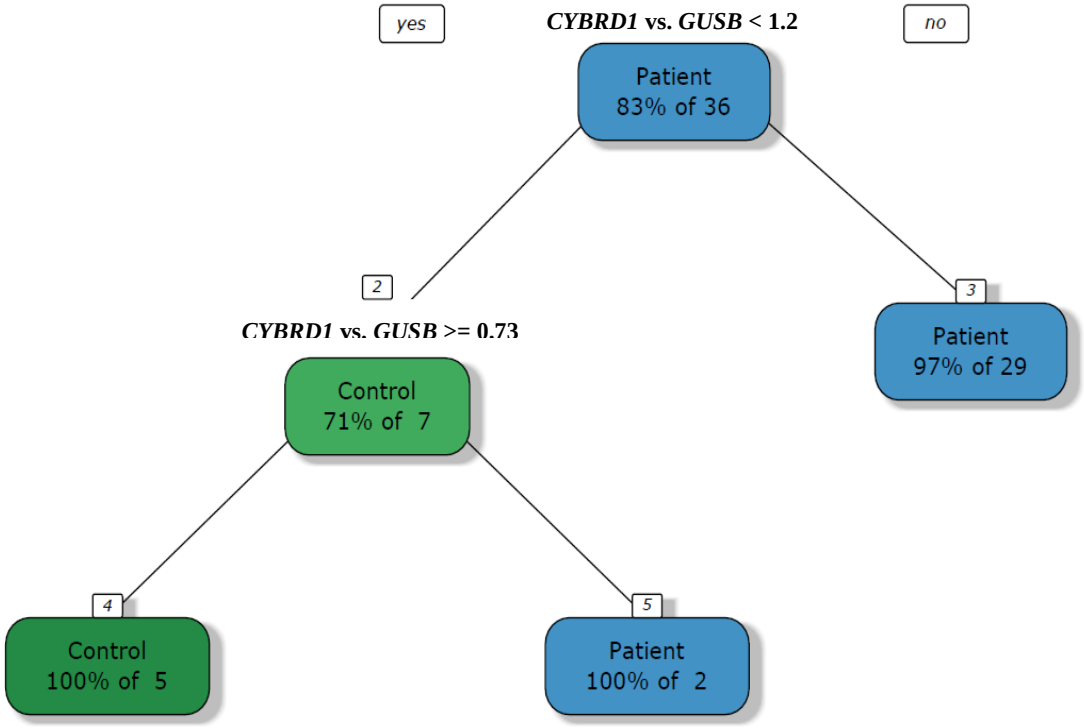

Figure 20 Decision tree showing the relative ratio cut-off point of 1.2 for *CYBRD1/GUSB*

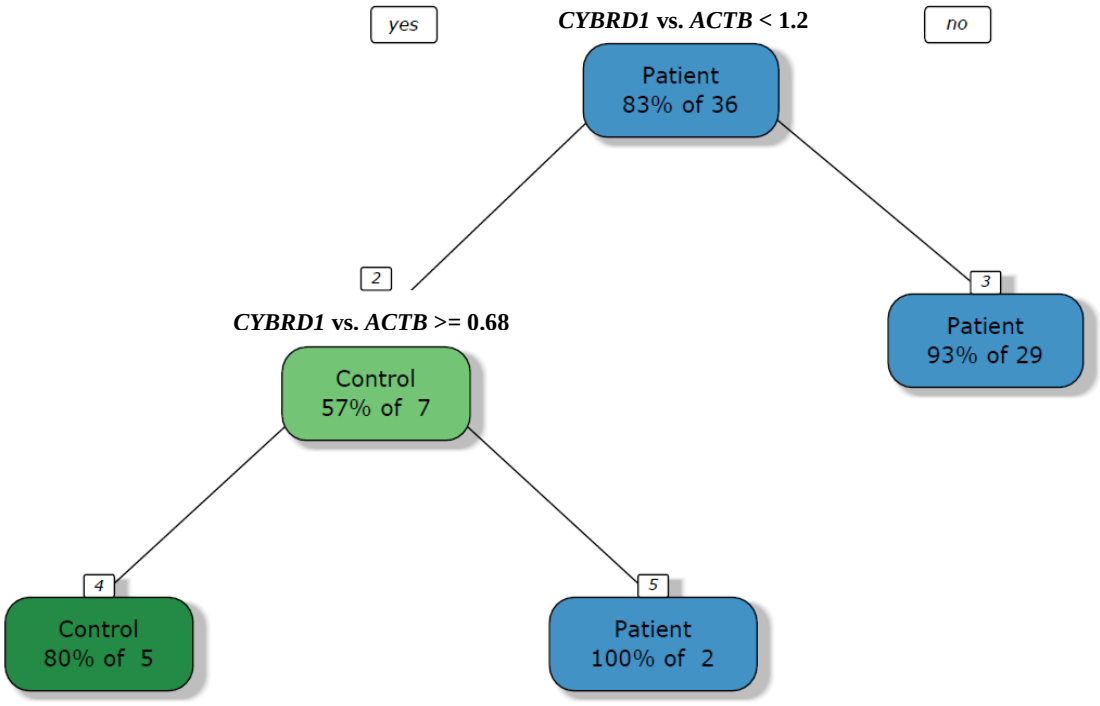

Figure 21 A significant cut-off value differentiating the study groups was determined as a ratio above/ below 1.2 for *CYBRD1/ACTB*

Decision tree analysis demonstrated that only *HMOX1* ratios, normalised to *GUSB*, demonstrated a significant cut-off value of 1.5 (Figure 22). 100% of 25 (TB patients) demonstrated ratios of greater than 1.5 while 55% of 11 (n=6) individuals categorised as controls displayed *HMOX1/GUSB* ratios less than 1.5. An odds ratio of 27.73 was calculated for individuals with a *HMOX1/GUSB* of greater than 1.5 of having TB, compared to individuals with *HMOX1/GUSB* expression below 1.5.

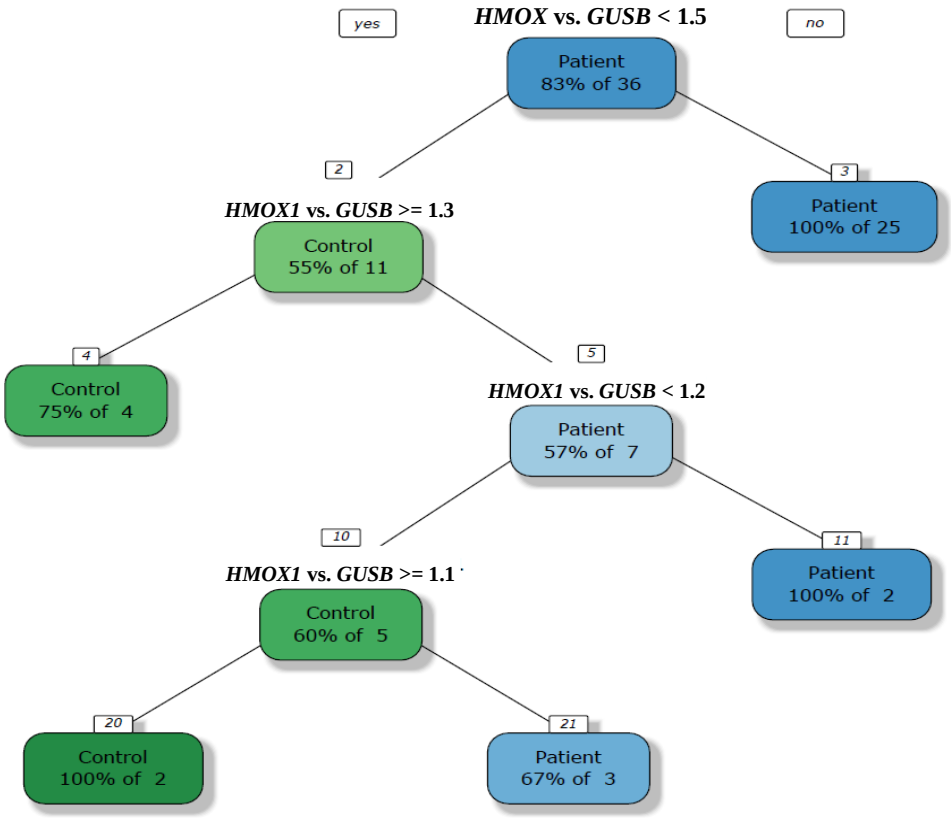

Figure 22 A relative expression ratio cut-off point of 1.5 is calculated *HMOX1* when normalised to *GUSB*

Expression ratios of *SLC40A1*, normalised to both *GUSB* and *ACTB*, demonstrated a significant cut-off value during decision tree and Fisher's exact test analysis ($p<0.05$). A slight difference in cut-off values was demonstrated as a ratio larger than 2 (normalised to *GUSB*) introduced a new node where 100% of 24 individuals were categorised as TB patients (Figure 23). 50% of 12 individuals (n=6) were controls and displayed a ratio less than 2. Ratios normalised to *ACTB* demonstrated that 100% of 21 individuals that were categorised as TB patients reported a ratio above 2.1 while 40% of 15 (n=6) individuals were controls and had ratios less than 2.1 (Figure 24). An odds ratio of 49 for *SLC40A1/GUSB* and 25.38 for *SLC40A1/ACTB* was calculated.

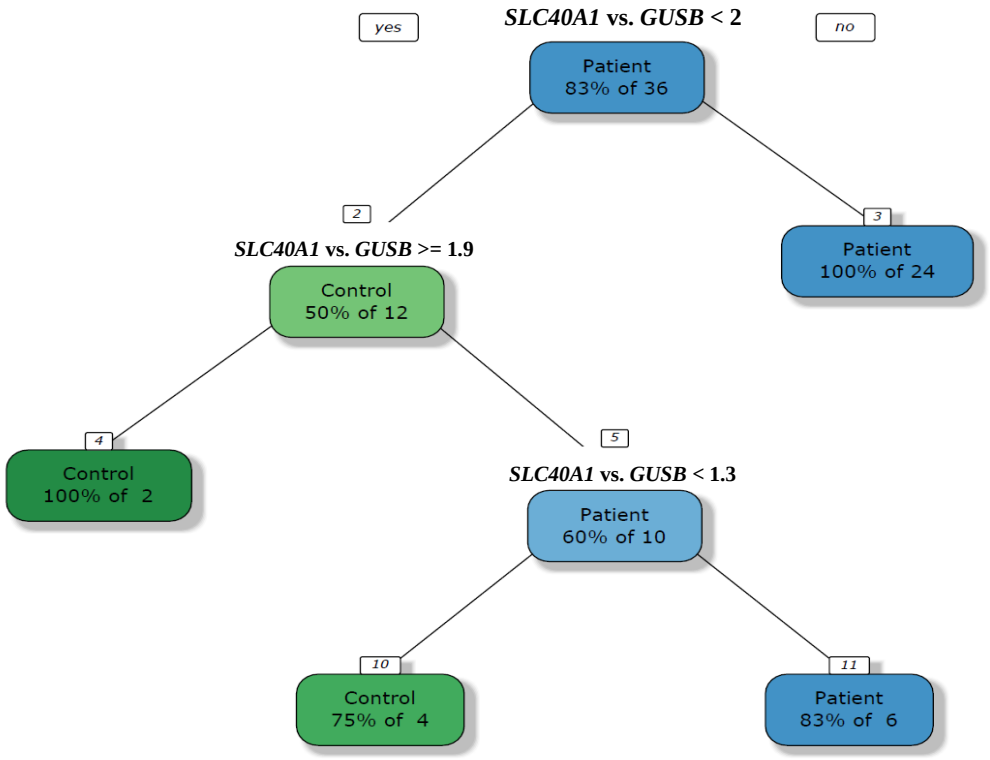

Figure 23 A relative expression ratio cut-off point of 2 is calculated for *SLC40A1* when normalised to *GUSB*

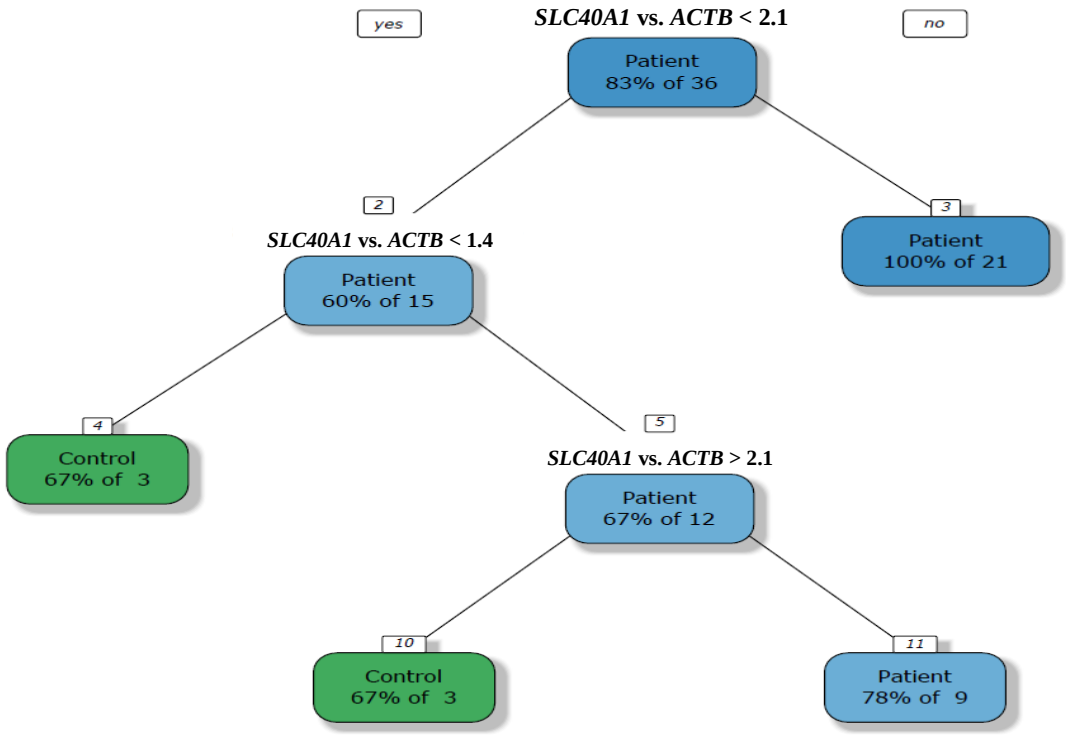

Figure 24 A significant cut-off value differentiating the study groups was determined as a ratio above/below 2.1 for *SLC40A1/ACTB*

3.4.8 Pearson correlations between genes

Measured transcript levels of a number of genes were correlated, with *SLC40A1* displaying the most correlations among the eight genes under investigation (Figure 25). *SLC40A1* displayed positive correlations with *FTH* (r = 0.67) and *FTL* (r = 0.59) ($p<0.005$). Understandably, the subunits of the protein required for iron storage demonstrated a positive correlation (r = 0.76) ($p<0.005$).

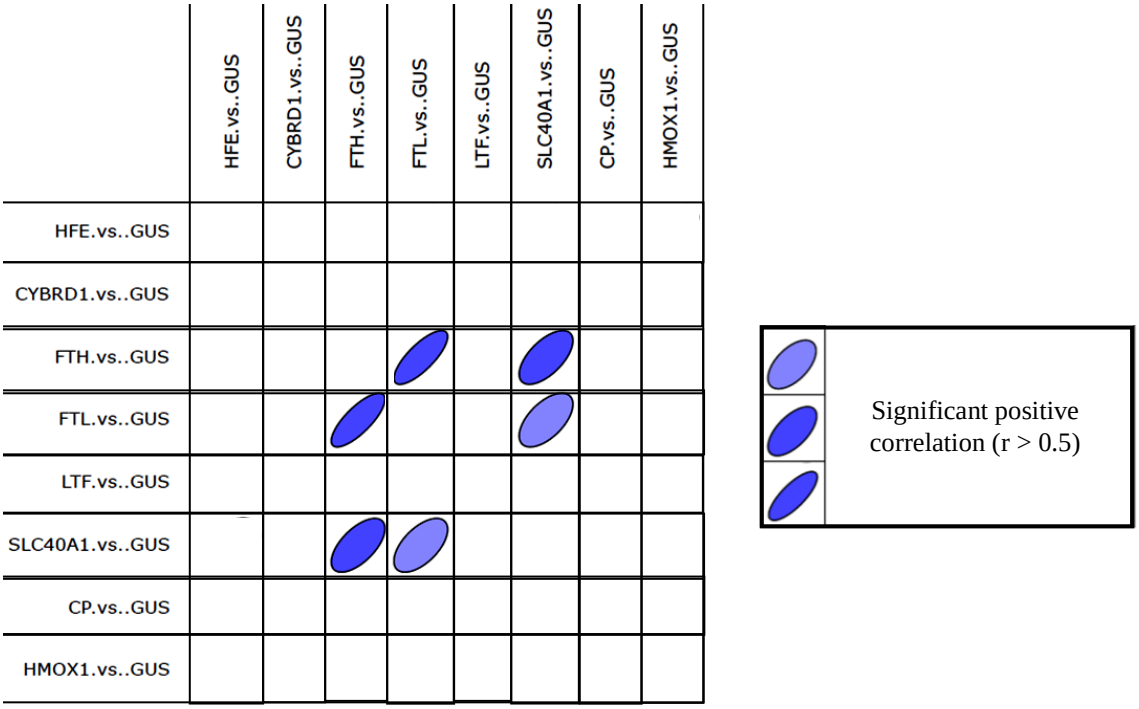

Figure 25 Correlation between eight genes of interest and *GUSB* using Pearson correlation

Pearson correlation coefficients were then determined for the gene genes under investigation when normalised to the *ACTB* reference gene. *HMOX1* now shows the most correlations among the genes. The gene pivotal in the iron-recycling process was positively correlated to *HFE* (r = 0.66) ($p<0.005$). *FTH* and *FTL* expression was positively correlated (r = 0.62) ($p<0.005$) (Figure 26).

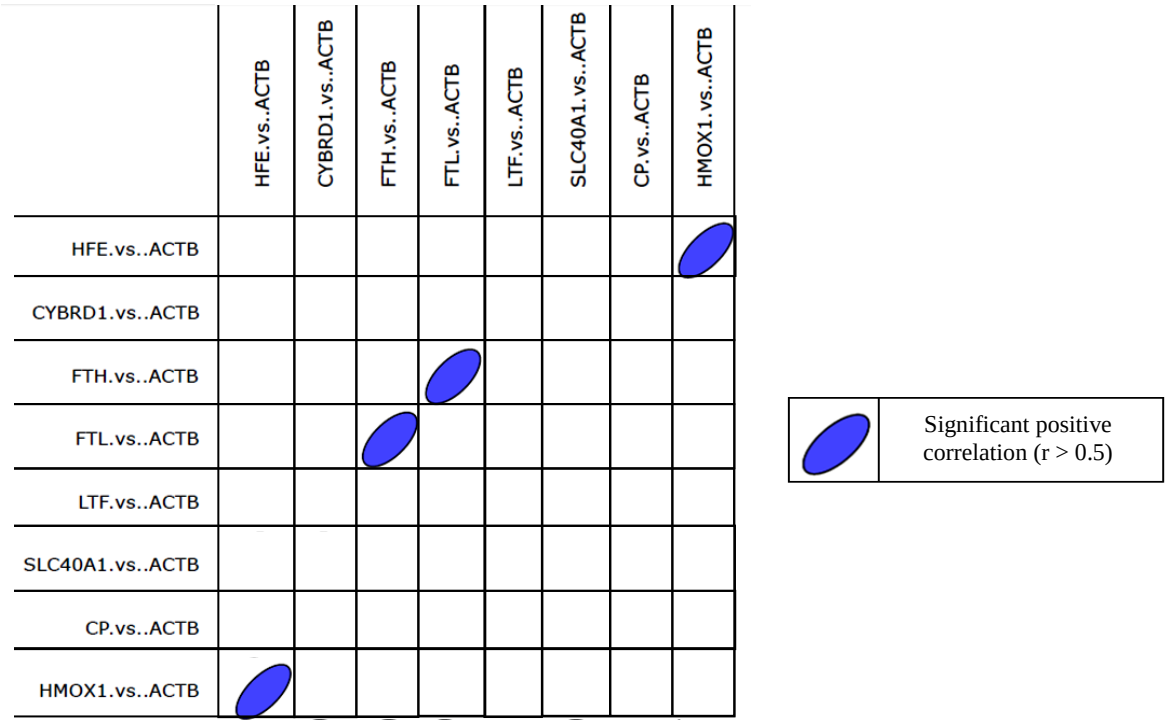

Figure 26 Correlation between 8 genes of interest and *ACTB* using Pearson correlation

Pearson correlation coefficients were then determined for the 8 genes under investigation when normalised to the *RPL37A1* reference gene. Significant positive correlations were observed between *HFE/RPL37A1* and *FTH/RPL37A1* ($r = 0.61$; $p < 0.005$), *FTL/RPL37A1* ($r = 0.75$; $p < 0.005$), *HMOX1/RPL37A1* ($r = 0.76$; $p < 0.005$), and *SLC40A1/RPL37A1* ($r = 0.67$; $p < 0.005$) (Figure 27). *FTH/RPL37A1* expression ratios are positively correlated with *FTL/RPL37A1* ($r = 0.85$; $p < 0.005$), *HMOX1/RPL37A1* ($r = 0.6$; $p < 0.005$) and *SLC40A1/RPL37A1* ($r = 0.81$; $p < 0.005$). Positive correlations were also seen between *FTL* and *HMOX1* ($r = 0.85$; $p < 0.005$), *LTF* ($r = 0.6$; $p < 0.005$), and *SLC40A1* ($r = 0.76$; $p < 0.005$).

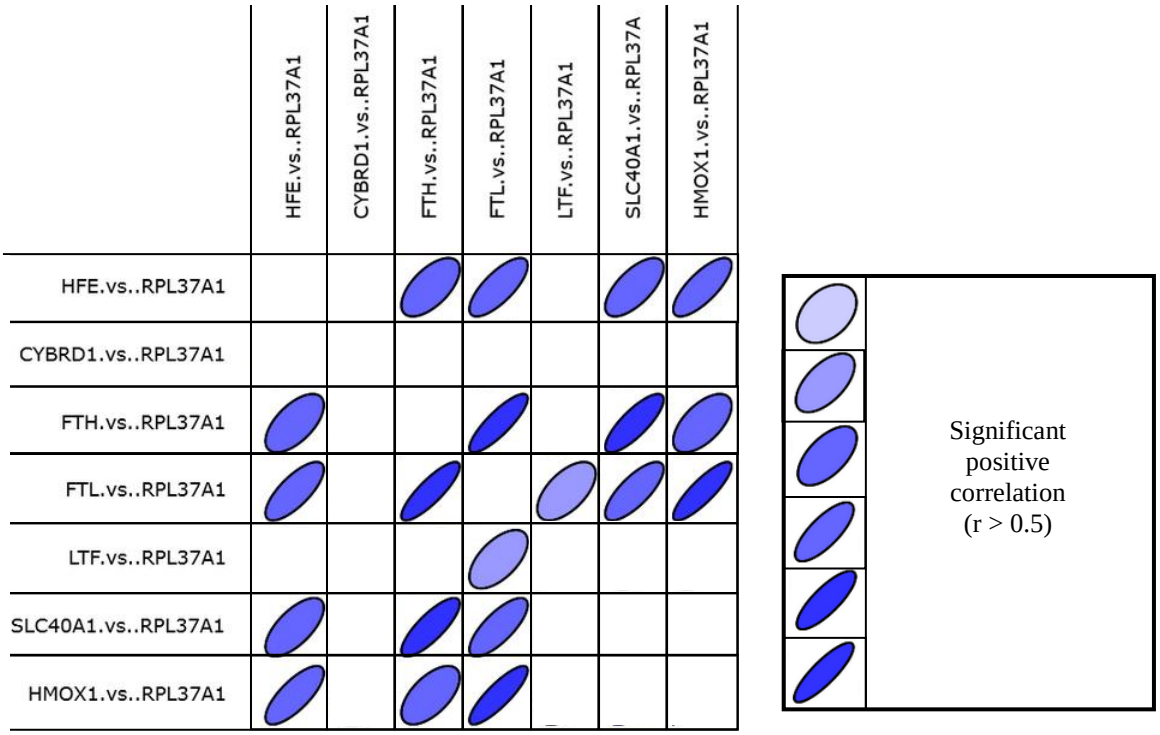

Figure 27 Correlation between eight genes of interest and *RPL37A1* using Pearson correlation

3.4.9 Iron parameters

Although a significance level of 5% was not reached, serum-FTN levels were elevated in the patient group (152.50 ± 33.00) with a *p*-value (0.08) approaching 5% compared to the control group (55.50 ± 26.25) (Figure 28 A). Significant differences in control and patient groups for iron (14.85 ± 1.90 and 11.15 ± 1.78) (Figure 28 B), STF (2.20 ± 0.20 and 2.44 ± 0.057) and TSAT (25.50 ± 2.25 and 21.30 ± 3.28) were not observed in this study.

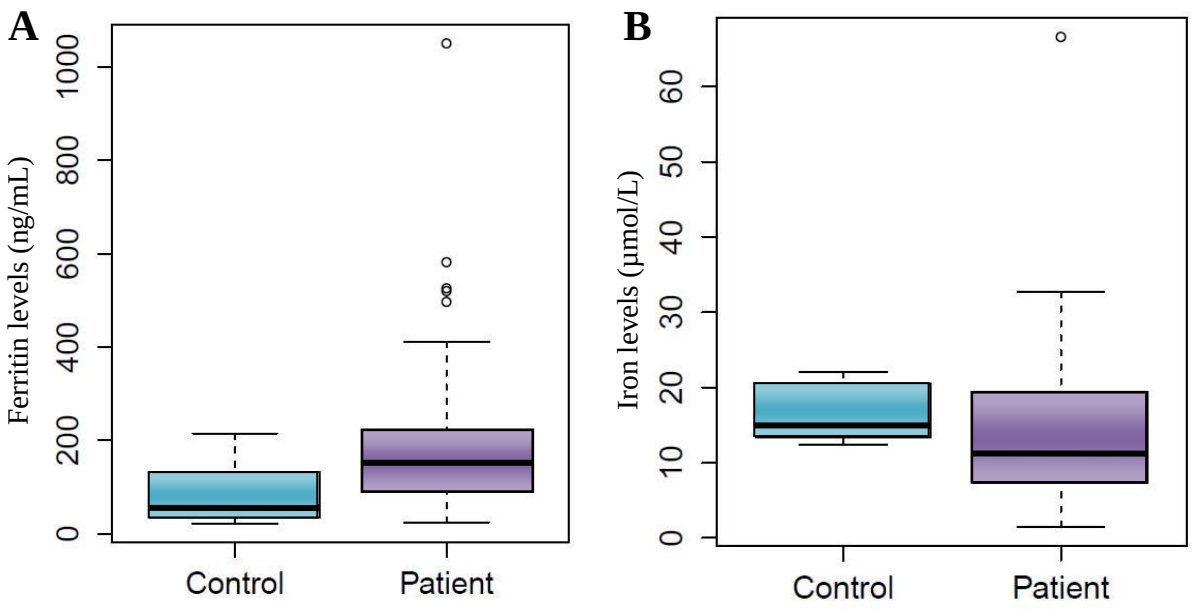

Figure 28 FTN levels demonstrated an increase between groups, serum-iron demonstrated a minor decrease, while STF and TSAT showed no differences between groups

A: Serum-FTN measurements for control vs. patient group. B: Serum-iron measurements for control vs. patient groups.

3.4.10 Immunology and liver-function parameters

Collectively, ten liver- and immune-specific parameters were measured and values were compared to investigate the possible differences that exist between control and patient groups. Significant differences were observed for total bilirubin ($p<0.01$), conjugated bilirubin ($p<0.05$), unconjugated bilirubin ($p<0.01$), total protein ($p<0.05$), and albumin ($p< 0.05$) levels (Figure 29).

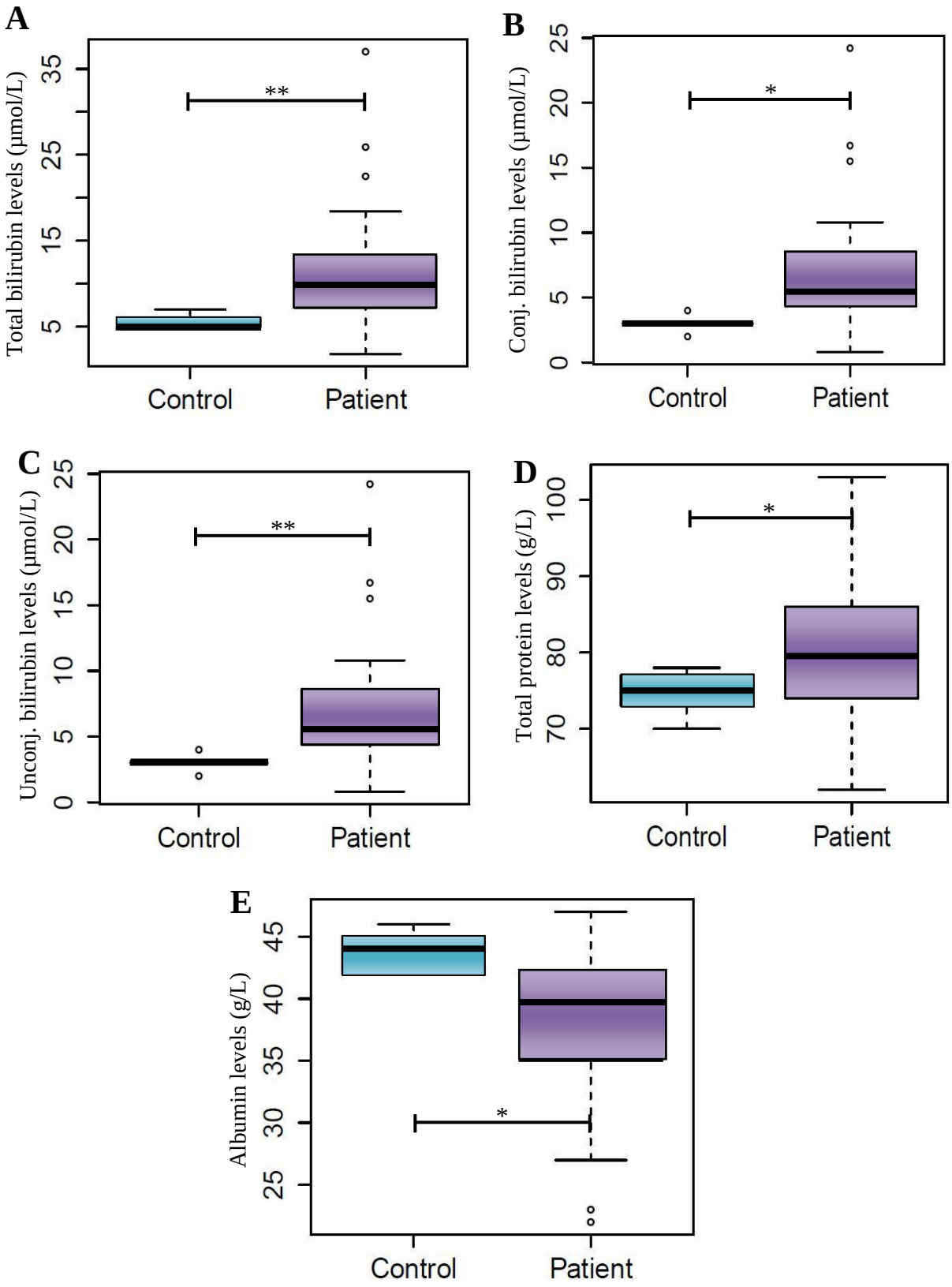

Figure 29 Elevated serum levels of total, conjugated, unconjugated bilirubin and total protein were recorded, while albumin was significantly lower in TB patients

A: Increased total bilirubin in TB patient group compared to control group ($^{**}p<0.01$). B: Increased conjugated bilirubin in TB patient group ($^{*}p<0.05$). C: Increased unconjugated bilirubin in TB patients in comparison to controls individuals ($^{**}p<0.01$). D: Elevated total protein in TB patient group ($^{*}p<0.05$). E: Decreased albumin levels in TB patient group compared to the control group($^{*}p<0.05$).

3.4.11 Pearson correlation between iron parameters and additional immunology and liver parameters separately

Correlations between the four iron parameters were then determined (Figure 30). The Pearson correlation tests between iron and FTN, TSAT and FTN, Tf and iron, and lastly, Tf and TSAT did not produce a significant positive or negative correlation. One significant negative correlation ($r = 0.62$) was observed between transferrin and FTN ($p<0.005$). Additionally, a positive correlation ($r = 0.98$) was shown between iron and TSAT in the study cohort ($p<0.005$).

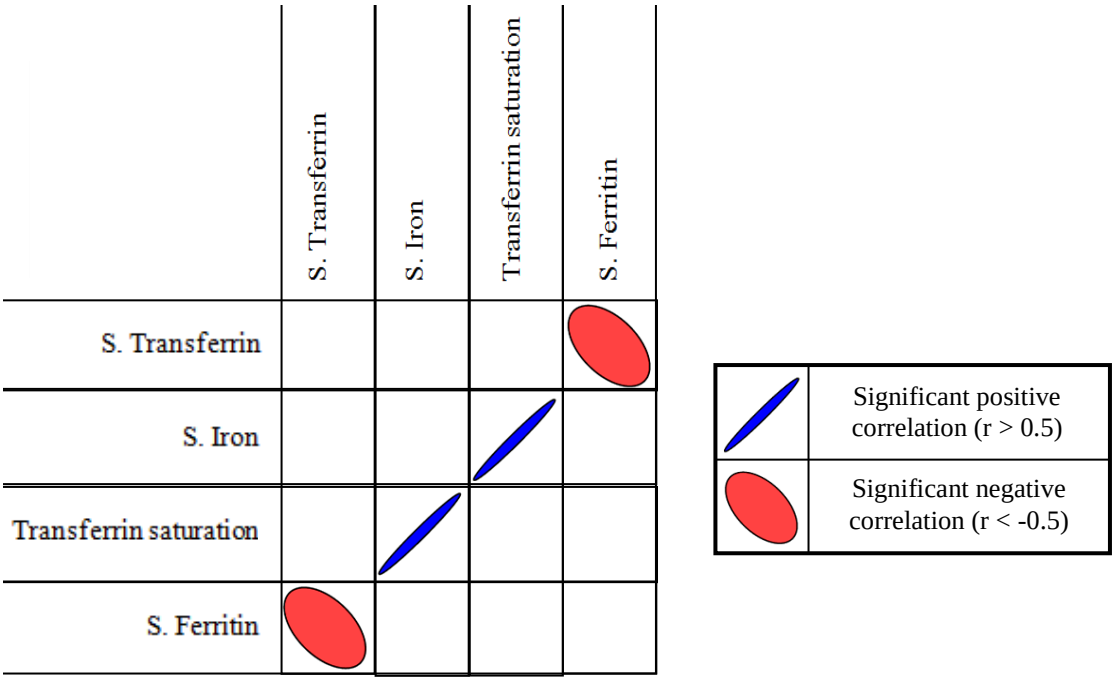

Figure 30 Significant correlations between STF and serum-FTN, and TSAT and serum-iron

Correlation analysis was subsequently employed to determine the existence of possible correlations that may exist between the one immunology parameter (CRP) and the liver-function parameters measured. Out of a possible 45 correlations possible between parameters, six coefficients were significantly difference from zero (Figure 31). The total protein and CRP parameters, did not show any significant correlations with other parameters with the exception of a negative correlation between albumin and CRP ($r = -0.81$; $p<0.005$). Understandably, total bilirubin is positively correlated to conjugated bilirubin ($r = 0.97$; $p<0.005$) and unconjugated bilirubin ($r = 0.99$; $p<0.005$). The unconjugated form of bilirubin positively correlated with conjugated bilirubin ($r = 0.93$; $p<0.005$). ALT showed positive correlations with GGT ($r = 0.71$; $p<0.005$) and AST ($r = 0.71$; $p<0.005$).

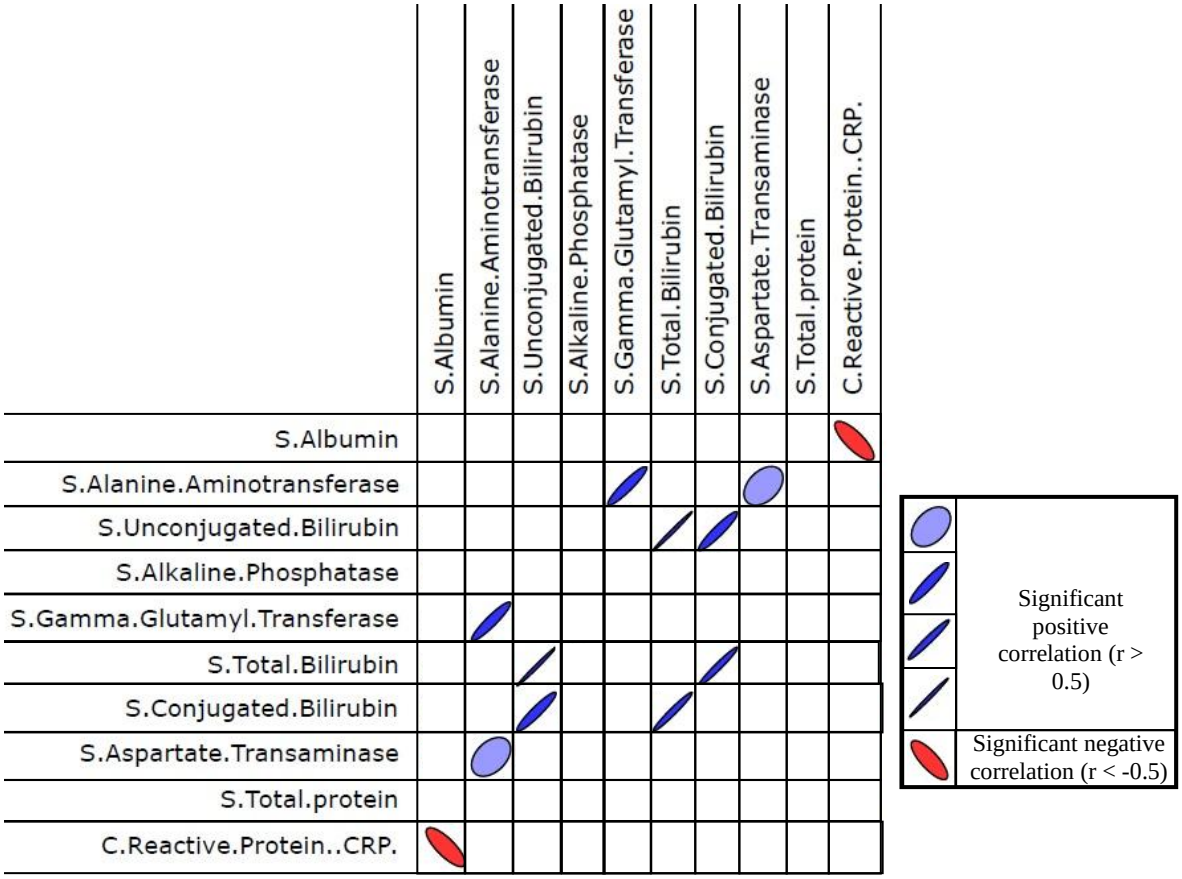

Figure 31 Correlation between 10 measured liver-function parameters using Pearson correlation

3.4.12 Association of low iron levels with differentially expressed *CP* and *HMOX1* ratios

We next investigated the associations that may exist between iron parameters and gene expression levels. Since 30% of patients presented with low serum-iron, these individuals were grouped (allocated as 1), while patients and controls with normal serum-iron were grouped (allocated as 0). *CP* and *HMOX1* expression relevant to *GUSB* was associated to low iron levels when groups were compared ($p<0.05$). In relation to *CP* expression, group 1 (0.63 ± 0.10) showed a significant decrease in comparison to group 0 (0.99 ± 0.07) (Figure 32 A), while a significant increase in *HMOX1* expression was noted between groups 0 (1.76 ± 0.20) and 1 (2.38 ± 0.17) (Figure 32 B).

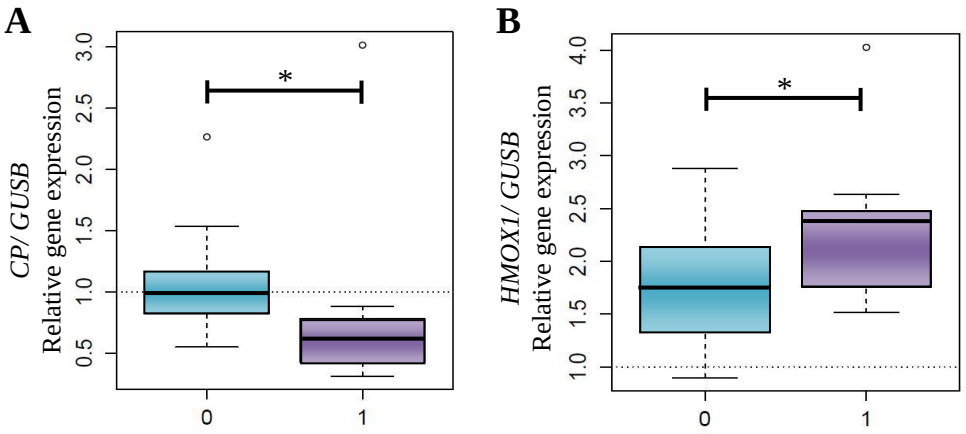

Figure 32 Association between low iron and decreased *CP* & increased *HMOX1* ratios

A: Box plot illustrates the significant association between study individuals with low serum-iron (allocated as 1) and decreased *CP* gene expression when compared to group allocated as 0 (*p<0.05). B: Box plot illustrating the association between individuals with low serum-iron (allocated as 1) and increased *HMOX1* expression in comparison to the group designated with a 0 (*p<0.05).

3.4.13 Association between low TSAT with *SLC40A1*, *FTL*, and *CP*

Furthermore, 40% of the patient group were characterised with low to very low TSAT and this parameter was then investigated for possible associations. Group 0 accounts for those study subjects with a normal to high TSAT, while group 1 denoted the study subjects with low to very low levels. Three genes (*CP*, *FTL* and *SLC40A1*) demonstrated significant associations ($p<0.05$). Decreased *CP* expression between group 0 (1.00 ± 0.05) and group 1 (0.73 ± 0.08) was associated with low TSAT (Figure 33 A) while an increase in *FTL* (0: 0.94 ± 0.05 and 1: 1.10 ± 0.04) (Figure 33 B) and *SLC40A1* (0: 1.98 ± 0.25 and 1: 2.99 ± 0.30) (Figure 33 C) expression was associated with low saturation levels.

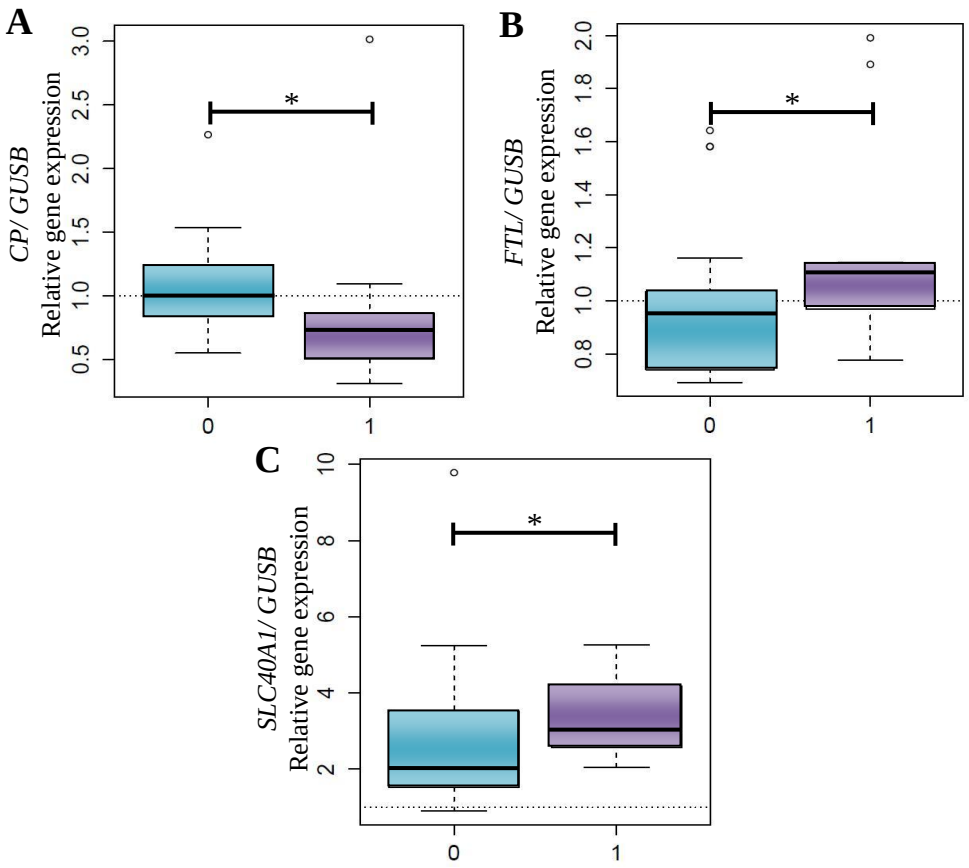

Figure 33 Association between decreased *CP* expression; increased *FTL* & *SLC40A1* expression is demonstrated with low TSAT

A: Box plot illustrates the significant association between study individuals with low TSAT (allocated as 1) and decreased *CP* gene expression (*p<0.05). B: Box plot illustrating the association between individuals with low TSAT (allocated as 1) and increased *FTL* expression (*p<0.05). C: Box plot illustrating the significant association between low TSAT and increased *SLC40A1* gene expression (p<0.05).

3.4.14 Gene expression profile summary

In summary, *CYBRD1*, *HFE*, *HMOX1*, and *SLC40A1* were significantly up-regulated in TB patients when compared to population-matched controls. Significant cut-off values and odds ratios for relative expression ratios for *CYBRD1*, *HFE*, *HMOX1*, and *SLC40A1* were calculated and show that certain gene profiles are associated with TB. *CP* expression was down-regulated when individuals were grouped according to iron status, demonstrating that *CP* may be iron-sensitive and not TB sensitive. Up-regulation of *FTL* requires further investigation and *HMOX1* and *SLC40A1* show iron-responsiveness. Reference gene selection and viability was ensured with *BestKeeper* software (Pfaffl *et al.*, 2004). No significant variations were demonstrated between control and patient groups in this study when Cq (also known as crossing point (CP) values) values were compared for all three reference genes utilised throughout the study (Figure B1 & Table B1).

Table 5 Summary table of gene expression profiles demonstrated for the TB group

Gene	Expression in TB group	Cut-off values	Expression with low serum-iron	Expression with low TSAT
CP	Unchanged	*N/A*	↓	↓
CYBRD1	↑	1.2	-	-
FTH	Unchanged	*N/A*	-	
FTL	Unchanged	*N/A*	-	↑
HFE	↑	1.2	-	-
HMOX1	↑	1.5	↑	-
LTF	Unchanged	*N/A*	-	-
SLC40A1	↑	2	-	↑

3.5 DISCUSSION

3.5.1 Elevated *CYBRD1* in TB group

Cytochrome b reductase is the primary ferric reductase responsible for the reduction of Fe^{3+} iron to Fe^{2+} iron, to accomplish iron uptake in the apical membrane of the duodenum, and expression is highly affected by iron status (Riedel *et al.,* 1995; Oakhill *et al.,* 2008). Experimental evidence from studies performed as early as 1964 show that dietary iron intake affects the rate of iron absorption (Pollack *et al.,* 1964). Researchers demonstrated that once rats were transferred from a diet containing high iron levels to one containing low iron levels, iron absorption increased (Frazer *et al.*, 2002).

In the current study, differing levels of sensitivity were obtained for *CYBRD1* when normalised to different reference genes but the overall trend in up-regulation remained consistent across the board (Figure 11 A, B & C). Also, odds ratios of 52.9 and 9.13 when *CYBRD1* was normalised to *GUSB* and *ACTB*, respectively, demonstrated that having TB is associated with increased *CYBRD1* levels compared to controls. Due to the fact that gene expression was not measured prior to and subsequent to infection, whether *CYBRD1* expression increased due to infection or it was initially increased and predisposed the individuals to TB infection, is currently unclear. The former explanation is expected as the overall response of the body to infection is to sequester iron (decrease bio-available iron) that would otherwise be available to pathogens for vital functioning and proliferation (Ward *et al.,* 2011). Although the body purposefully reduces systemic iron concentrations to combat infection, *CYBRD1* may become induced to increase iron absorption to compensate for altered iron levels (Frazer *et al.,* 2002). This could explain the noted increase in *CYBRD1* expression shown in TB patients if the *CYBRD1* expression increased after TB infection. Alternatively, if *CYBRD1* expression was elevated before TB infection, larger volumes of iron would have been available to the infectious pathogen to assist growth since this gene induces iron absorption in the duodenum (Riedel *et al.,* 1995; Oakhill *et al.,* 2008). Although this explanation is mechanistically correct, further studies that measure iron within hepatocytes and duodenal enterocytes should be performed to determine if the elevated *CYBRD1* expression and associated increase in iron absorption resulted in increased systemic iron or whether it was held in enterocytes or stored in the liver.

Although gene expression was elevated in the TB group compared to the controls, individuals with low iron levels did not demonstrate the same increase compared to individuals with normal serum-iron. This finding was unexpected as research shows a direct relationship between *CYBRD1* expression, iron absorption and iron status (Zoller *et al.*, 2003). A further look at the significant correlation between iron and TSAT (%) documented in this study does correspond to evidence that Frazer and colleagues presented in 2002. This group suggested that TSAT (%) can be deemed a regulatory factor, which indicates iron-overload or deficiency. This finding was explained by the fact that Tf both binds iron during absorption and transports this element to several tissues throughout the body (Hirose, 2000). Therefore, a diet deficient in iron (low systemic iron) correlates to decreased TSAT, which in turn, should directly signal cells to increase the expression of *CYBRD1* to facilitate iron absorption to restore iron balance. Additional support is derived from studies led by Cox and Peters (1978) and Zoller *et al.* (2003) that demonstrated an increase in *CYBRD1* mRNA and protein expression in patients that presented with iron deficiency. Increased expression was explained by the body's compensatory mechanism employed to counteract low serum-iron by actively increasing iron absorption. Alternatively, increased *CYBRD1* expression that is measured prior to TB infection could act as a susceptibility factor to *M. tuberculosis* infection due to augmented iron absorption. Increased iron absorption could result in overall iron increase, which needs to be stored in FTN at the intracellular level to prevent toxic effects of iron. Macrophages also express FTN to store excess iron and the accumulation of iron-bound FTN in response to increased overall iron in macrophages could provide an important source for continued *M. tuberculosis* growth.

For non-disease patients, the same mechanism would come into play, but findings from this study do not show a difference in *CYBRD1* profiles for individuals with low iron. The 10 individuals characterised with decreased serum-iron levels were also diagnosed with TB, which could account for the failure of *CYBRD1* gene expression induction to increase iron absorption. It is therefore postulated that individuals with decreased iron levels will continue to present with low levels since the body cannot utilise the above mentioned mechanism to increase serum-iron. In relation to individuals with TB infection and low serum-iron, iron levels may remain diminished as the expected increase in *CYBRD1* expression necessary to curb iron depletion appears not to be active in these individuals. As iron facilitates the innate immune response to pathogen invasion, individuals that cannot activate the required

induction of *CYBRD1* to increase iron absorption, may demonstrate an increased susceptibility to TB infection, due to an impaired immune response.

3.5.2 Storage genes remain unaffected following TB infection

Harrison and Arosio (1996) determined that both the heavy- and light- subunits of FTN collectively bind excess iron to prevent the formation of reactive species; they also serve as a reservoir that can be utilised by the body during times of depletion. Therefore, the iron status of the body directly effects the production of FTN molecules.

mRNA levels of both subunits demonstrated no significant differential expression between patient and control groups in the current study (Figures 12 A & B; Figure 13 A & B), with the exception of ratios normalised to *RPL37A1* (Figures 12 C & 13 C) that demonstrated up-regulation. This finding is explained by the regulatory mechanism that governs the expression of these subunits. Both chains possess IRE motifs that reversibly bind IRP molecules when iron concentrations are either high or low (Hentze & Kuhn, 1996). This post-transcriptional modification pattern explains the unaffected transcript levels observed. However, further research focusing on the expression of protein products of both subunits is vital in understanding the relationship between FTN, iron and TB susceptibility since FTN levels in the patient group were significantly higher than the control group (Figure 28 A). Additional support for continued examination of this relationship comes from work presented by Miller *et al.* (1991) that showed a distinctive pattern of FTN up-regulation in response to cytokines released during inflammation associated with infection. Both tumour necrosis factor and interleukin-2 (IL-2) initiate an increase in FTN protein expression in macrophages and hepatocytes as part of the inflammatory cascade following infection. The response to IL-2 did not have an effect on mRNA levels but induced protein expression *via* specific protein interactions at a region rich in quanosine-cytidine repeats present in the mRNA sequence (Rogers *et al.*, 1994).

Moreover, correlation analysis from the current study indicate that serum-FTN levels correlate in a negative manner with STF, which is understandable as an environment of high iron would result in increased FTN production to aid storage, while Tf levels would decline as iron is not needed by the body if supply is high (Figure 30). As previously mentioned, research aimed at measuring the serum-FTN and protein expression levels before and after TB infection might determine whether elevated FTN documented in this study was caused by

the inflammation response or the host's attempt to decrease iron availability only, or whether elevated FTN predisposes an individual to infection. The latter explanation could derive from an initial response to bind iron and decrease access of this element to the pathogen, which also resulted in decreased iron required by macrophages for appropriate destruction of pathogenic bacilli.

3.5.3 Gene expression during iron transport *via* LTF

LTF was isolated from the milk of cattle in 1960 during unrelated research conducted by Groves, Johanson and Montreuil and colleagues. Amino acid sequence similarities noted between Tf and LTF led researchers to believe that both proteins share similar functions (Bluard-Deconinck *et al.*, 1974; Jollés *et al.*, 1976). In addition to the ability of LTF to bind iron, the degree of infection noticeably reduced in mice when the protein was administered. This role during infection is explained by the protein's capacity to sequester iron during states of infection to reduce bio-available iron for pathogenic growth (Schaible *et al.*, 2002).

Differential *LTF* expression was not observed in the TB and control groups in the current study and no significant changes were seen between those individuals presenting with low serum iron compared to those presenting with normal serum-iron levels (Figure 15 A, B & C). Furthermore, no significant correlations between *LTF* and the other genes under investigation were established. Expression ratios below 1 (normalised to all three reference genes) were recorded for *LTF*, which can be explained by the low expression of both mRNA and protein levels in serum. The highest levels of LTF are present in milk and bodily secretions (Groves, 1960; Johanson, 1960; Montreuil *et al.*, 1960; Masson *et al.*, 1966). Although *LTF* expression remained unaffected during the current study, research published by several groups point to the role of this protein product in antimicrobial activity during the immune response to TB infection. Evidence for these antibacterial properties comes from cell culture experimentation during which the metabolism of carbohydrates was affected in *Streptococcus mutans* cells (Arnold *et al.*, 1982). In addition, a research group reported that LTF played a protective role against *Escherichia coli* infection other than primarily sequestering iron (Zagulshi *et al.*, 1989). Further research should therefore measure *LTF* and LTF levels in tissues with high expression profiles for LTF, including macrophages in the lungs. *M. tuberculosis* sequesters iron from LTF more readily than Tf and measuring the change in *LTF* and LTF expression in the lungs could lead to the identification of levels associated to an increased susceptibility to TB.

3.5.4 Iron export gene is elevated in TB patients

FPN is the protein on the baso-lateral membrane of duodenal and macrophage cells that primarily facilitates iron efflux (Beaumont, 2010). Work performed by Motley and colleagues (2004) provides evidence that a fundamental interaction between infection, hepcidin expression and FPN levels exists. An increase in IL-6 expression corresponds to an increase in hepcidin expression following bacterial invasion. An inverse relationship between hepcidin and FPN levels results in a noted decrease in FPN expression, subsequently resulting in hypoferremia. On a molecular level, hepcidin binds to membrane-bound FPN proteins resulting in FPN internalization and ultimate degradation (Delaby *et al.*, 2005). A decrease in FPN-mediated iron export directly results in reduced systemic iron associated with infection. A reduction in systemic iron is beneficial to the host during infection as decreased bio-available iron leads to a decline in pathogen growth and proliferation.

This sequence of events is not documented in the current study since *M. tuberculosis* infection has not decreased *FPN* expression in TB patients (Figure 16 A, B & C). Expression levels deduced from *ACTB* reference gene highlighted *FPN* as the most significant distinguishing factor between the two study groups (Figure 24).This finding may indicate that individuals presenting with increased *FPN* expression before being exposed to the bacterium have an increased risk of developing TB. A possible explanation for this finding stems from research performed on different macrophage populations that demonstrated differential expression of *FPN* and its protein product (Van Zandt *et al.*, 2008). The significant increases in *FPN* expression in certain cell lines could be explained by whether macrophage cell lines are un-stimulated or stimulated by *M. tuberculosis* infection and IFN-γ, as well as the basal expression of *FPN* in each cell line. Peritoneal macrophages (low basal *FPN* expression) demonstrate increased transcript expression, while bone marrow derived macrophages and lung macrophages (higher basal *FPN* expression) show the exact opposite. Further protein analyses on peritoneal macrophages by this group show no significant increase in protein expression. A reason for the unexpected protein result is derived from research into the translational regulation of this gene (Liu *et al.*, 2002). This research shows that the 5' upstream untranslated region of *FPN* contains an IRE that is governed by iron status in the body. During conditions of low iron related to infection, the expression of FPN decreases significantly due to the presence of the well-understood IRE-IRP system at the upstream position.

Further studies that investigate the basal level of *FPN* expression could aid in our understanding of whether the increase in *FPN* expression reported in this study is due to low basal expression levels or whether consistently high expression prior to infection made certain individuals more susceptible to infection. Results in this study showed increased *FPN* expression when individuals with low TSAT levels were compared to individuals with normal levels (Figure 33 C). Because individuals with low TSAT status all presented with TB, previous research dictates that FPN expression should decrease according to the inverse relationship with hepcidin (described above), which is not seen on the genetic level. Protein analysis can determine whether high *FPN* expression (current study) translates into elevated FPN expression or whether the IRE-IRP system described for *FPN* holds true when iron concentrations are altered in the body. Data published by several groups that documented FPN expression patterns warrant consideration in terms of the current study. These research groups describe the pattern of FPN mobilization to either the phagosome membrane following pathogen phagocytosis, or the macrophage membrane when old erythrocytes are engulfed for ultimate degradation and recycling (Chung *et al.*, 2004; Knutson *et al.*, 2005). Increased levels prior to infection could affect macrophage function since iron is continuously exported out of the immune cell, thereby reducing its bactericidal activity predisposing these individuals to infection. Because low iron levels reduce FPN expression, individuals that present with low serum-iron prior to infection could result in decreased iron export from phagosomes once bacterium are phagocytosed by macrophages.

3.5.5 Ferroxidase expression in the bloodstream remains unchanged

The primary function of ceruloplasmin in the plasma is to oxidise ferrous iron to ferric iron to facilitate the binding of this crucial element to the transporter protein- Tf (Curzon & O'Reilly, 1960). The current study demonstrated that *CP* transcript levels were not differentially expressed between controls and TB patients (Figure 14 A, B & C). However, when groups were differentiated on the basis of low serum-iron levels and TSAT instead of TB infection, *CP* expression was significantly decreased in the low iron and TSAT groups compared to the group with normal levels (Figures 32 A & 33 A). These findings correspond to findings that display a relationship between iron and copper deficiency resulting in an increase in *CP* expression. More recently, work published in 2002 investigated the relationship between *CP* levels in the liver and circulating ceruloplasmin levels and found that a significant decrease or increase in iron status did not correspond to alterations in hepatic *CP* levels or systemic ceruloplasmin (Tran *et al.*, 2002). Although significant

differential expression was not documented between overall groups, a combination of low serum-iron or TSAT and decreased *CP* expression may result in these individuals demonstrating an increased susceptibility to active TB infection. As the primary aim of the host's defence is to sequester iron to decrease bio-availability to the pathogen, hypoferremia ensues. Research by two unrelated groups shows that when CP levels are diminished, iron starts to accumulate (Frieden, 1970; Wirth & Linder, 1985). Increased iron levels provide the perfect resource for continued bacterial growth and, therefore, these individuals might show an increased susceptibility to infection.

3.5.6 Augmented *HFE* expression in TB patients

The *HFE* gene was identified less than 20 years ago and research into its function, localization, expression and regulation has become the topic of major research groups around the world (Feder *et al.*, 1996). The crystal structure of the HFE protein shows that the protein exhibits a sequence similar to Tf thereby allowing the protein to bind to membrane-bound TfR (Bennett *et al.*, 2000). HFE binds to TfR on the membrane of duodenal cells, macrophages, and monocytes to significantly decrease the receptor's affinity for iron-bound Tf when intracellular iron levels are elevated (Parkkila *et al.*, 2000).

Data presented in this study show an increased *HFE* expression in TB patients in comparison to population-matched controls for all three reference genes employed (Figure 17 A, B & C). Decision tree analysis indicated that a *HFE/GUSB* ratio of above 1.2 introduced a specific differential node between patient and control individuals, demonstrating the significant difference in expression levels measured (Figure 19). In relation to the *GUSB* reference gene, HFE is the most distinguishable factor between the control and patient groups. In other words, the 1.2 level of expression was shown to be the most significant deciding factor to whether a sample would be classified as a control or a patient. The node created within the decision tree, with the first split being an expression level less than 1.2, showed that 3 out of 3 samples that met this requirement were control samples. A certain level of caution should be taken when decision trees are analysed since the current study utilised a small sample. Furthermore, a positive correlation between *HFE* and *HMOX1* transcript levels was observed (Figures 26 & 27). The comparison of *HFE* levels in individuals with low iron to those with normal levels showed no significant difference. This finding was unexpected since iron status influences the expression of *HFE* to either increase or decrease iron uptake in cells. Evidence for this association is provided in a research paper published shortly after *HFE* was

discovered (Riedel *et al.,* 1999). A significant decrease in iron uptake *via* TfR and stimulation of IRP activity, resulting in FTN mRNA degradation, is illustrated by this group using HeLa cells programmed to express high levels of *HFE*. Current data cannot confirm this finding as TfR expression was not monitored and FTN expression was measured prior to any post-transcriptional (mRNA degradation) modifications taking place.

Several research groups show that *in vivo* expression of HFE is predominantly expressed within intestinal cells and liver macrophages to facilitate cellular iron uptake (Parkkila *et al.,* 1997; Bastin *et al.,* 1998). Patients suffering from HH (HFE deficiency) should therefore demonstrate elevated iron levels in intestinal cells and liver macrophages due to the absence of functional HFE proteins to regulate iron uptake. Conversely, these target cells in HH sufferers exhibit iron levels similar to those reported for iron deficiency as a result of inappropriately augmented IRP activity (Cairo *et al.,* 1997). This data conflicts research that illustrates that increased *HFE* results in increased IRP activity and FTN mRNA degradation (Riedel *et al.,* 1999). Future work to fully establish the relationship between iron, HFE expression, and IRP activity is important since conflicting results inhibit the use of pre-symptomatic testing in relation to HFE levels. Regardless of whether increased or decreased HFE results in FTN mRNA breakdown, inappropriate FTN production results in the dys-regulation of iron storage in HH individuals. Excess iron (not sequestered by FTN) is shifted to liver cells and these cells become damaged and later replaced with fibrous tissue (Arthur, 1996). These findings, although contradictory, indicate that iron plays an important role in HFE expression. However, results from this study do not demonstrate a significant difference between individuals with normal and low iron levels. This is unexpected as a number of studies link iron and HFE expression. Therefore, an alternative factor may play a role in the host response to TB infection. Evidence for hepcidin as the proposed factor comes from *Hfe* knockdown experiments in mice (Wallace *et al.,* 2011). A combination of elevated HFE and hepcidin levels is required to achieve iron-withholding mechanisms employed by the host defence against *M. tuberculosis.*

3.5.7 Gene expression during iron recycling

HMOX1 and the by-products of haem degradation (iron, biliverdin, and CO) play a protective role during inflammation and excessive cytokine release in several cell types (Kyokane *et al.,* 2001; Otterbein *et al.,* 2003; Kravets *et al.,* 2004; Nakao *et al.,* 2004).

Research shows that an elevation in *HMOX1* expression acts to shield the liver from the acute production of cytokines released during inflammation (Yeligar *et al.*, 2010).

The data analysed in the current study show a significant elevation in *HMOX1* expression in TB individuals when compared to population-matched controls (Figure 18). A significant correlation was noted between *HFE* and *HMOX1* (Figures 26 & 27). When study subjects were separated based on iron status, individuals with low iron showed a significant increase in *HMOX1* transcript levels. These results were anticipated as several lines of evidence signify the role of HMOX1 in the protection against mycobacterial infection in mice and rat models (Silva-Gommes *et al.*, 2013). Suliburk and colleagues (2009) utilised a rat model (subjected to LPS-inducted liver damage) to examine the protective effects of HMOX1 against liver injury. Once injury was induced, HMOX, COX-2, and iNOS protein levels were examined. Results indicated a significant increase in HMOX expression, while the opposite was found with regards to COX-2 and iNOS protein levels. These two proteins play a crucial role in the inflammatory response and are therefore important in the host's accumulative response against infection. A continued decrease in expression may lessen the host's innate response towards pathogen invasion, thereby favouring the continued proliferation of that pathogen. The build-up of haem in *Hmox1*$^{-/-}$ mice proves to be cytotoxic in macrophages that do not express the all-important, rate-limiting enzyme of erythrocyte catabolism (Silva-Gommes *et al.*, 2013). Furthermore, Regev and colleagues (2012) demonstrated the vital role HMOX1 has during the formation of granulomas following the phagocytosis of mycobacterium. Elevated levels of Hmox1 (mouse form) shows an increase in iron release from macrophages to ensure that excess iron within cells do not cause apoptosis in mice (Ferris *et al.*, 1999). The opposite holds true since mice with diminished levels of Hmox1 accumulated intracellular iron and cellular death ensued. This research provides direct evidence for the relationship between iron status and *HMOX1*.

The positive correlation between *HFE* and *HMOX1* is expected because the host sequesters iron away from macrophages, the primary site of *M. tuberculosis* proliferation. Both proteins are increased during infection to limit iron uptake and promote iron efflux. The increased *HMOX1* expression in individuals with low iron levels is contradictory to literature mentioned above. According to the current understanding of HMOX1 function, elevated intracellular iron levels are attenuated *via* increased iron efflux from macrophages, which in turn should result in elevated systemic iron. Two limitations of the current study could

explain this finding, because as intracellular iron was not measured, the entire HMOX1 response cannot be determined. In addition, long periods of infection could result in iron storage in the liver (not measured here) that could account for low systemic iron measurements. Although HMOX1 and the reaction by-products are beneficial to the host, elevated *HMOX1* expression prior to TB infection would result in high intracellular iron. Elevated levels would result in a larger iron pool from which the microbe could sequester iron for growth, thereby predisposing these individuals to TB infection.

3.5.8 Correlations between iron parameters

The current study demonstrated a significant negative correlation between transferrin levels and measured FTN levels. Furthermore, a significant positive correlation was recorded between serum-iron and Tf saturation percentage (Figure 28). TSAT is calculated as the percentage of iron that is bound to transferrin in the serum, therefore an exact positive correlation is expected. The body's reaction to low FTN levels is to increase iron absorption *via* the duodenal enterocytes. An increase in iron absorption results in increased levels of bio-available iron that can bind to Tf in the bloodstream and can be transported to those cells that require this vital element.

3.5.9 Immunology and liver-function parameters

Bilirubin, the by-product of the haem catabolism, is insoluble in water and therefore requires a transporter protein to enter into the blood stream. This lipid-soluble, hydrophobic form of bilirubin is referred to as unconjugated bilirubin and binds to serum-albumin in reversible fashion to gain entry into the circulatory system. Once the complex is transported to the space of Disse it separates to facilitate the transport of bilirubin into hepatocytes. Through a series of cytosolic protein and enzymatic activity in the liver cell, bilirubin is conjugated to glucoronic acid, which is hydrophilic. Conjugated bilirubin is subsequently released into the bile for excretion by the body (Pratt, 2010).

Data presented in this study illustrate a significant increase in both forms of bilirubin (collectively known as total bilirubin) in TB patients when compared to controls (Figure 29 A, B & C). Since the unconjugated form binds to albumin to gain entry into hepatic target cells, the measured albumin levels were significantly attenuated in TB patients, i.e., increased amounts of albumin were bound to unconjugated bilirubin resulting in lower free albumin in the serum (Figure 29 E). Furthermore, significant positive correlations were demonstrated

between both forms of bilirubin and total bilirubin (Figure 31). This finding is expected since total bilirubin is merely the sum of both forms and an increase or decrease in unconjugated bilirubin will directly affect the amount of conjugated bilirubin that can enter the hepatocytes. Research demonstrates that bilirubin levels are elevated during host infection (Franson *et al.*, 1985). This research group shows that 88% of individuals recruited for the study presented with hyperbilirubinemia. According to previous research, elevated levels of bilirubin are associated with mycobacterial infection, which is consistent with data presented in this study. Several lines of evidence described unconjugated bilirubin as a powerful antioxidant that protects the host against excessive damage caused by reactive species production (Minetti *et al.*, 1998). More recently, research demonstrated that excessive levels of unconjugated bilirubin could intensify the process of infection (Kapitulnik, 2004; Khan & Poduval, 2011). Since bilirubin demonstrates both cyto-protective and cyto-toxic properties, a delicate balance should be maintained and the inclusion of this liver-function test should be incorporated in the clinical panel of tests to determine TB susceptibility.

Current data show that total protein levels are significantly augmented in TB patients (Figure 29 D). No further correlations are demonstrated between this parameter and others measured in this study. Total protein is the measure of two categories of protein present in the serum namely albumin and globulin. A substantial increase in total protein in TB patients cannot be attributed to an increase in albumin since this parameter (serum-albumin) demonstrates a significant reduction in the current study. Alternatively, the significant increase may occur from an elevation in globulin levels (not measured here). This finding is concordant with research published as early as 1953, in which researchers showed a significant increase in globulin levels in TB individuals when compared to control individuals (Gillem & Newman, 1953).

ALT and AST have successfully been used to diagnose liver injury in certain cases, while liver biopsies are recommended when these levels remain normal or elevated for long periods and other signs of liver disease start to appear (Oh & Hustead, 2011). Both enzymes facilitate the movement of an amino group present in alanine and aspartic acid to the keto group in ketoglutaric acid (Pratt, 2010). Since AST is present in a multitude of tissue types throughout the body and ALT is predominately found within the liver, ALT is considered the more important indicator of liver disease. Correlations between the remaining parameters demonstrate two significant positive correlations between ALT and GGT, and ALT and AST

and one negative correlation between CRP and albumin. Neither of the transaminase levels were significantly elevated in TB patients recruited in this study. Liver damage is the most common reason for elevated ALT and AST in the serum and the absence of differences between study groups may indicate that TB patients do not present with liver damage. Low to normal serum-iron measured here may indicate that iron is not being loaded in the hepatocytes, i.e. iron-induced liver damage is not recorded. The positive correlation observed in this study merely indicates that the two enzyme function alongside one another and an increase in one parameter will most often be associated with an increase in the other and *vice versa*.

GGT is present on the cell membrane of several tissue types, including the liver, pancreas, spleen, and kidneys and occurs in the serum of healthy individuals (Pratt, 2010). Although GGT is requested by a physician during liver-function determination, the non-specific nature of GGT curbs its significance in the clinical setting. When utilised in combination with ALT and AST levels, GGT can aid in the diagnosis of liver disease, and increases in ALT (primarily expressed in liver cells) and GGT are anticipated due to the clinical application these two parameters share.

The final correlation noted in this study is the negative correlation observed between CRP and albumin. Low albumin levels (shown here also) are demonstrated in individuals with liver and kidney disease (Pratt, 2010). CRP is present in high levels in individuals that have an infection. Therefore, a negative correlation would exist between CRP and albumin levels as high levels of CRP and low levels of albumin both indicate the presence of infection.

Chapter 4

CONCLUSION AND FUTURE PROSPECTS

4. CONCLUSION AND FUTURE PROSPECTS

The ultimate aim to experimentally measure several iron gene expression levels in patients that present with active TB and population-matched controls to establish significant correlations between iron parameters, gene expression and a possible increase in susceptibility to developing active TB infection was achieved. Several attempts were made at optimizing the PCR conditions for *CP*, *HAMP*, *HEPH*, *SLC11A1*, and *SLC11A2*, with success demonstrated for *CP*. Accurate standard curves were optimised for genes under investigation (*CP*, *CYBRD1*, *FTH*, *FTL*, *HFE*, *HMOX1*, *LTF*, and *SLC40A1*) and reference genes (*ACTB*, *GUSB*, and *RPL37A1*). The expression levels of all eight genes of interest were determined relative to corresponding standard curves. Normalised ratios were computed to determine differences in expression between TB and control groups and the correlations among several iron, immunology and liver-function parameters were investigated. Parameter measurements were also compared between TB and control groups to identify significant differences.

An increase in *CYBRD1* expression prior to TB infection could result in the storage of excess iron in FTN in macrophages, which can be sequestered by *M. tuberculosis via* multiple pathways for growth and proliferation. Elevated *HFE* levels would result in attenuated iron absorption through the traditional iron-bound Tf-TfR mechanism on cell membranes. Although an overall decrease in iron absorption through this mechanism would decrease, *M. tuberculosis* will not suffer from decreased iron availability as the microbe shows a much higher preference for obtaining iron from iron-bound LTF. Since *LTF* and LTF are primarily expressed in milk and bodily secretions, differences between TB patients and controls were not expected. However, LTF acts as a potent antimicrobial protein during infection and alterations in this gene and encoded protein can point to differences in TB susceptibility. It is therefore proposed that samples be collected from lung tissue to quantify levels in the macrophages that initially interact with *M. tuberculosis.* Augmented *HMOX1* levels observed in the TB group may protect the host against oxidative damage caused during inflammation. However, the presence of elevated levels before TB infection would result in increased bio-available iron for *M. tuberculosis* growth in macrophages since HMOX1 breaks down erythrocytes to release iron. TB patients presenting with low TSAT show decreased *CP* expression which was anticipated as less iron is in the oxidised form which can freely bind to Tf in the plasma. Increased transcript levels of *SLC40A1* before TB infection could

predispose individuals to infection since elevated amounts of iron are exported from cells that express FPN, resulting in an increase in plasma iron, which could bind to LTF or Tf and gain access into macrophage cells. Although transcript levels were not significantly different between study groups, measured serum-FTN showed a substantial increase in TB patients. This discordant expression pattern is due to the action of the post-transcriptional regulation of FTN mRNA *via* the IRE-IRP system and iron status. Individuals demonstrating increased FTN levels could be at-risk for TB infection as FTN increases in hepatocytes, intestinal cells, and macrophages in response to increased iron absorption and global iron increases. Increased FTN expression and subsequent iron binding in macrophages could result in an increased iron pool from which invading *M. tuberculosis* can sequester iron for growth.

4.1 IDENTIFIED LIMITATIONS

As in most research, several limitations are present which require consideration prior to future endeavours. Several short-comings are listed below, requiring the consideration of research groups investigating the correlations that may exist between differential expression of iron genes and increased/decreased susceptibility to TB infection.

4.1.1 Cohort HIV status is not documented

Human Immunodeficiency Virus (HIV) and TB both activate the host immune response although different cascades are stimulated and different immune cells respond to each foreign pathogen. Depleted CD4+ T cells (present in HIV+ individuals) could drastically influence gene expression profiles or the overall response to the TB pathogen. Consent was not provided to incorporate the HIV status of study participants during data collection.

4.1.2 Knowledge of current illness

This limitation is listed since the lack of study cohort infection status is one of the most significant drawbacks: the elevation of several gene expression profiles cannot necessarily be attributed only to TB infection. Although CRP levels (serum measurement of infection) were not significantly elevated in the cohort, this does not exclude the possibility that several individuals may have presented with infection during the period of whole blood collection.

4.2 FUTURE PROSPECTS

Research focusing on the gene and protein expression modifications related to TB susceptibility could include the following aspects to strengthen reproducibility and promote the use of expression profiling in the clinical setting.

4.2.1 Investigate expression in several tissue types

Iron status is regulated by absorption that primarily occurs in the duodenal section of the intestine and iron export from hepatocyte stores in the liver. Additionally, the breakdown of senescent erythrocytes largely occurs within macrophages located in the spleen. To examine the effect of TB infection on iron regulators, several tissues should be sampled to determine which type should be utilised in the clinical setting to provide the most accurate expression pattern. Although, the analysis of genetic or parameter profiles using tissue biopsies is not always easily accessible and convenient as a diagnostic tool.

4.2.2 Sample a larger cohort that is well-characterised

To increase statistical significance and representations of the population, a larger cohort of an equal number of control and patient groups should be sampled as randomly as possible. The incorporation of drug resistance, current and past therapeutic interventions, history of disease, and HIV/AIDS status should prove vital in drawing conclusions from expression profiles. Mutation screening of genes should also be carried out to determine if expression is as a result of genetic aberration or iron status. The names and quantities of anti-TB medication should be recorded to classify patients accordingly to certain drug regimes.

4.2.3 Incorporation of several additional iron regulators and immune markers

The optimization and expression quantification of the other iron regulatory genes that were not completed in this study including *HAMP*, *HEPH*, *SLC11A1*, and *SLC11A2* could prove to be insightful in understanding the complex relationship between iron status, immune function, and TB susceptibility. The determination of IL-6, IFNγ, NO, ROI, and RNI levels (markers of immune response) requires consideration in future.

4.2.4 Iron-macrophage staining

Measured iron levels were only recorded once for each study individual and each value only represents the amount of serum-iron. All the observations and correlations in this study were made using serum-iron levels, which is often not the same throughout all the cells in the

body. Tissue specific iron staining in macrophages could indicate the intracellular level of this vital element and aid in understanding the regulation of iron during TB infection.

4.3 SUMMARY

Study objectives were met with the exception of optimization conditions for *HAMP*, *HEPH*, *SLC11A1*, and *SLC11A2*. Generated gene expression profiles for control and TB-positive individuals were compared and differential expression patterns and correlations among profiles and measured parameters were identified. Overall, aberrations in the gene expression and serum levels of iron regulators in TB groups were documented throughout the study. Although the data presented require corroboration, it points to the importance of including these genes and iron parameters in a panel of tests to determine an individual's risk of developing active TB in the absence of other confounding factors, such as liver disease, hemochromatosis, and HIV infection.

Chapter 5

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5. REFERENCES

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APPENDICES

Appendix A- Summary of cDNA conversions and concentration used in the study

Table A1 Number of rounds and the use of 1X or 10X cDNA for each gene

Gene Abbreviation	Full gene name	No. of rounds performed	Conc.(C)/diluted(D) cDNA used
CP	Ceruloplasmin gene	2	C
CYBRD1	Cytochrome b reductase gene	3	D
FTH	Ferritin heavy Chain gene	3	D
FTL	Ferritin light Chain gene	3	D
HFE	Haemochromatosis gene	3	D
HMOX1	Haem oxygenase 1 gene	3	D
LTF	Lactotransferrin gene	3	D
FPN/SLC40A1	Ferroportin gene	3	D
ACTB	B-Actin gene	2	C (only compared to *CP*)
		3	D
GUSB	β-Glucuronidase gene	2	C (only compared to *CP*)
		3	D
RPL37A1	Ribosomal protein L37	3	D

Appendix B- Validation of references genes

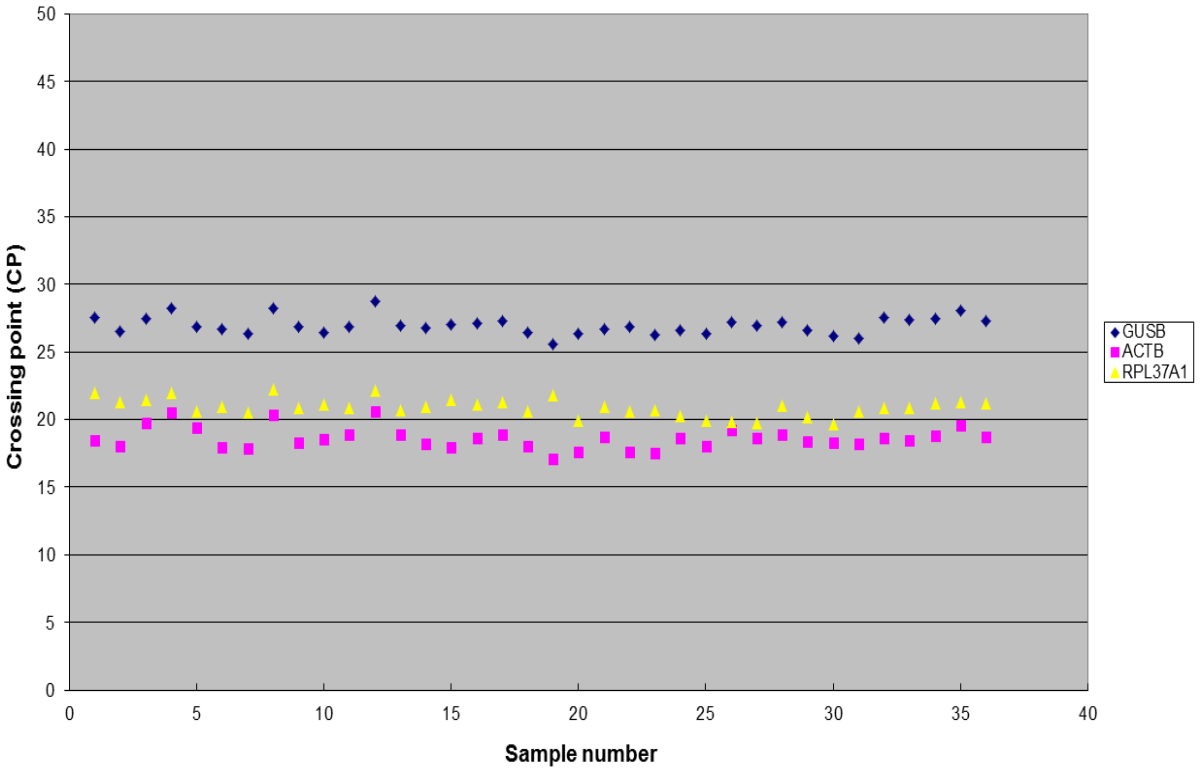

Figure B1 Calculation CP variation in reference genes (*GUSB*, *ACTB* and *RPL37A1*)

BestKeeper software was utilised to determine the variation of reference genes within the cohort. No significant variations were observed (Pfaffl *et al.*, 2004).

Table B1 Descriptive statistics (mean, minimum, maximum and standard deviation) of reference genes

Descriptive Statistic	Reference Gene		
	GUSB	***ACTB***	***RPL37A1***
n	36	36	36
Geometric Mean [CP]	26.964	18.614	20.899
Arithmetic Mean [CP]	26.972	18.631	20.908
Minimum [CP]	25.616	17.067	19.666
Maximum [CP]	28.746	20.600	22.206
STD [± CP]	0.512*	0.592*	0.492*

Abbreviations: *ACTB*= beta-actin gene; CP= crossing point; *GUS*= beta-glucuronidase gene; n= sample size; *RPL37A1*= ribosomal protein L37 gene; STD= standard deviation

* Standard deviation values below 1 reveal that the gene is suitable as a reference gene (Pfaffl *et al.*, 2004).

Appendix C- Raw average Cq and relative concentration values

Table C1 Summarised table of average calibrated, relative ratios obtained when combing data from round 1, 2, and 3 for *HFE*, *CYBRD1*, *FTH* and *FTL*

Sample number	*HFE*			*CYBRD1*			*FTH*			*FTL*		
	GUSB	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*	*RPL37A1*
TB02	3.287	1.868	6.264	3.31	1.76	6.21	2.20	1.16	4.20	1.58	0.87	3.02
TB04	0.990	0.779	2.247	1.48	1.22	3.43	1.88	1.47	4.30	1.12	0.88	2.54
TB05	0.783	1.082	1.233	1.45	2.16	2.11	0.90	1.32	1.36	0.94	1.32	1.35
TB06	0.614	0.824	0.773	1.67	2.23	2.03	1.52	2.03	1.89	1.01	1.34	1.25
TB07	0.854	1.620	0.985	1.56	2.48	1.86	0.58	0.91	0.68	0.55	1.09	0.64
TB08	3.256	2.229	5.542	0.38	0.26	0.69	1.01	0.68	1.77	1.10	0.74	1.90
TB09	1.191	0.977	1.951	2.83	2.19	4.41	1.32	0.98	1.96	1.00	0.85	1.50
TB10	1.764	2.277	2.687	3.21	3.89	5.15	0.99	1.29	1.47	0.78	1.04	1.13
TB11	2.765	3.664	11.418	1.27	1.63	4.37	0.86	1.16	3.33	1.10	1.46	3.82
TB12	2.222	1.697	3.041	2.63	1.93	3.80	1.51	1.22	2.24	1.58	1.21	2.12
TB13	1.370	0.912	1.829	3.43	2.28	5.10	1.33	0.88	2.20	1.06	0.71	1.10
TB14	1.882	1.275	2.634	2.93	1.98	4.07	1.94	1.27	2.89	1.13	0.81	1.70
TB15	1.405	1.383	1.918	2.74	2.53	3.60	0.87	0.79	1.19	0.74	0.67	0.97
TB16	2.608	2.325	5.850	2.86	2.36	5.80	0.83	0.69	1.93	1.10	0.91	2.38
TB17	2.337	1.648	3.304	4.48	3.39	6.19	1.35	1.03	1.89	1.46	1.12	2.07
TB18	2.068	2.469	4.656	1.33	1.54	2.90	0.78	0.89	1.66	0.97	1.11	2.05
TB19	1.441	1.645	2.094	2.46	2.85	3.59	0.89	1.01	1.26	0.74	0.84	1.06
TB20	1.448	1.778	1.800	2.06	2.36	3.73	0.72	0.79	0.72	0.69	0.75	0.68
TB21	1.339	1.512	1.648	2.96	3.30	3.60	0.60	0.67	0.71	0.71	0.79	0.84
TB22	1.504	1.130	2.398	2.11	1.58	3.29	1.21	0.88	1.87	1.04	0.76	1.60
TB23	2.337	1.269	4.337	2.12	1.16	4.18	1.61	0.87	2.72	1.89	1.03	3.54
TB24	1.421	1.172	2.590	2.36	1.96	4.49	0.76	0.62	1.34	0.75	0.67	1.39
TB25	1.284	1.210	1.963	3.93	3.34	5.71	1.14	0.97	1.67	0.97	0.82	1.40
TB27	1.813	1.468	2.723	3.75	3.04	5.67	0.91	0.74	1.24	0.89	0.72	1.34
TB32	0.846	0.626	5.096	0.54	0.40	3.22	0.66	0.48	3.90	0.92	0.67	5.34
TB43	3.303	2.267	3.597	4.47	3.04	4.73	0.73	0.50	0.79	1.03	0.70	1.09
TB46	2.542	2.466	3.653	1.84	1.68	2.50	0.80	0.96	1.38	0.70	0.83	1.19
TB47	2.771	1.348	3.342	2.28	1.11	3.14	2.21	1.07	2.72	1.99	0.97	2.38
TB48	1.953	1.347	3.724	3.36	2.32	6.32	1.10	0.73	2.05	1.14	0.77	2.14
TB49	1.803	2.067	1.798	1.44	1.66	1.51	0.87	0.99	0.86	0.82	0.95	0.79
TC01	0.905	0.860	0.877	1.20	1.15	1.15	1.10	1.00	1.06	1.03	0.96	0.98
TC02	0.776	0.853	0.363	1.09	1.16	0.53	0.81	0.89	0.39	0.96	1.09	0.48
TC03	0.675	0.704	0.453	1.00	0.96	0.63	0.82	0.88	0.57	1.13	1.18	0.77
TC04	0.885	1.004	2.242	2.25	2.23	4.79	0.95	1.00	1.85	0.98	1.03	2.30
TC05	1.023	1.123	1.527	0.92	1.02	1.28	0.70	0.77	0.94	0.73	0.78	1.04
TC06	0.930	1.179	1.025	1.20	1.57	1.38	0.85	1.12	0.98	0.94	1.25	1.09

Table C2 Summarised table of average calibrated, relative ratios obtained when combining data from round 1, 2, and 3for *LTF*, *SLC40A1*, *HMOX1* and *CP* (only 2 rounds performed in this case)

Sample number	*LTF*			*SLC40A1*			*HMOX1*			*CP*	
	GUSB	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*	*RPL37A1*	*GUSB*	*ACTB*
TB02	3.77	2.15	7.33	9.78	5.52	18.64	1.54	0.92	2.95	1.52	1.09
TB04	1.69	1.33	3.87	2.74	2.15	6.13	1.54	1.21	3.47	0.40	0.40
TB05	1.34	1.05	3.08	1.09	1.57	1.57	1.63	2.13	2.26	1.06	2.02
TB06	0.11	0.17	0.16	2.37	3.15	2.92	0.96	1.31	1.17	0.96	1.26
TB07	0.30	0.40	0.37	2.26	3.69	2.61	1.06	1.69	1.23	1.20	2.49
TB08	0.09	0.14	0.11	2.48	1.68	4.34	1.52	1.03	2.63	3.01	2.82
TB09	2.25	1.76	3.46	3.50	2.76	5.30	1.22	0.97	1.90	0.67	0.71
TB10	0.56	0.76	0.78	3.26	4.30	4.76	2.11	2.75	3.16	1.09	1.87
TB11	0.48	0.65	1.65	2.37	3.13	7.82	2.63	3.48	9.15	0.63	1.29
TB12	0.93	0.63	0.71	5.23	4.00	7.28	2.25	1.71	3.05	0.86	0.61
TB13	0.40	0.27	0.55	3.57	2.37	4.89	1.24	0.83	1.83	0.78	0.53
TB14	1.00	0.79	1.56	3.81	2.56	5.27	2.38	1.61	3.35	0.99	0.70
TB15	0.70	0.61	0.80	2.67	2.46	3.58	2.17	1.93	2.75	0.73	0.65
TB16	0.31	0.25	0.65	2.72	2.23	6.00	2.48	2.03	5.12	0.68	0.53
TB17	0.16	0.12	0.21	3.92	2.94	5.49	1.93	1.47	2.67	0.72	0.66
TB18	1.13	1.35	2.46	2.82	3.27	6.02	2.45	2.84	5.29	0.31	0.52
TB19	1.69	1.93	2.45	1.82	2.07	2.59	1.90	2.16	2.73	0.60	0.85
TB20	2.00	2.16	1.98	5.22	5.64	4.55	1.71	1.91	1.75	2.02	2.42
TB21	0.17	0.19	0.20	1.88	2.09	2.24	2.03	2.31	2.50	0.90	1.29
TB22	0.14	0.10	0.22	3.53	2.60	5.45	1.75	1.24	2.67	0.55	0.53
TB23	0.26	0.15	0.52	4.92	2.66	8.99	4.25	2.29	7.95	0.43	0.27
TB24	0.46	0.38	0.76	1.68	1.40	3.19	1.97	1.63	3.69	1.06	1.16
TB25	0.34	0.28	0.49	5.27	4.51	7.63	2.38	2.00	3.49	0.88	1.10
TB27	0.64	0.52	0.98	3.49	2.83	5.23	2.63	2.13	4.04	1.35	1.55
TB32	8.52	6.27	50.35	2.05	1.51	12.04	1.73	1.29	10.37	0.58	0.57
TB43	7.83	5.34	8.45	1.96	1.34	2.12	2.66	1.82	2.85	1.46	1.26
TB46	0.57	0.81	1.16	1.56	1.80	2.60	2.39	2.75	3.98	1.28	1.49
TB47	0.34	0.17	0.41	4.87	2.38	5.81	2.29	1.12	2.80	0.84	0.53
TB48	15.75	11.25	31.76	3.16	2.17	5.81	2.06	1.43	3.98	0.78	0.73
TB49	0.48	0.55	0.46	1.41	1.62	1.38	2.17	2.48	2.16	1.54	2.22
TC01	1.21	1.11	1.24	1.89	1.87	1.75	1.15	1.07	1.11	1.07	1.37
TC02	0.62	0.69	0.30	1.89	2.08	0.94	1.12	1.33	0.54	1.03	1.37
TC03	0.35	0.36	0.25	1.21	1.25	0.85	1.43	1.51	1.01	0.99	1.20
TC04	0.58	0.61	1.65	1.84	2.01	4.00	1.42	1.49	3.05	1.01	0.76
TC05	1.01	1.04	1.53	0.90	0.98	1.23	0.90	0.98	1.31	1.13	0.87
TC06	0.50	0.67	0.58	1.16	1.53	1.34	1.54	2.01	1.78	0.91	0.87

Appendix D- RT-PCR gel and standard curve

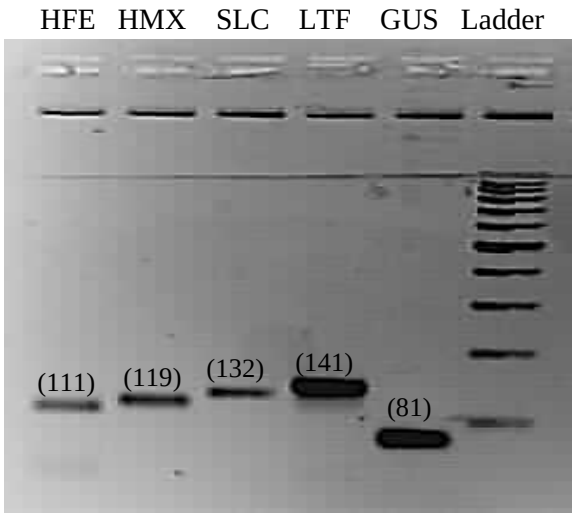

Figure D1 Gel photo of *HFE*, *HMOX1*, *SLC40A1*, *LTF* and *GUS* to confirm that the band of interest was accurately amplified accordingly to expected fragment length in bp

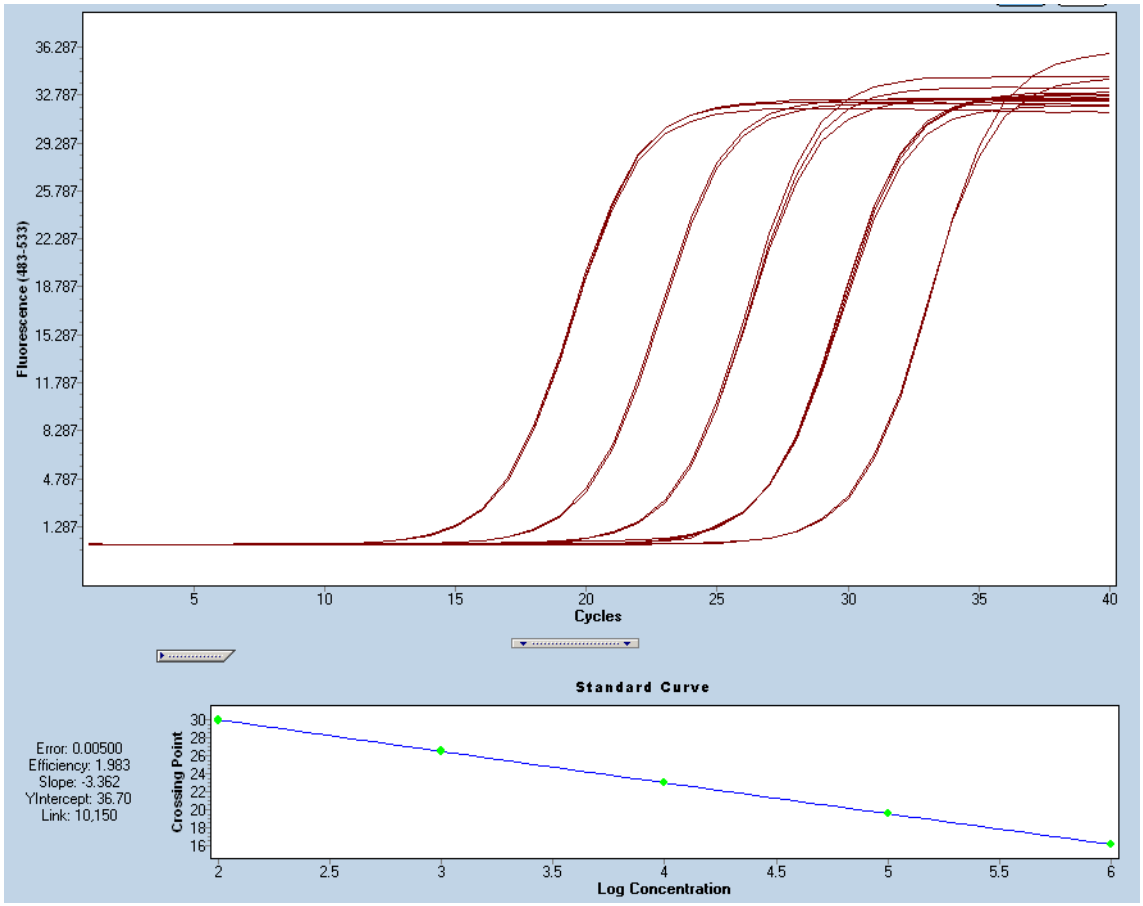

Figure D2 Standard curve generated for *HFE* indicating accurate efficiency and slope values

Appendix E – RNA integrity ratios

Table E1 260/280 Ratios measured during concentration determination

Sample number	260/280 Ratio	Sample number	260/280 Ratio
TB02	2.08	TB21	2.12
TB04	2.05	TB22	2.15
TB05	2.10	TB23	2.07
TB06	2.03	TB24	2.07
TB07	1.98	TB25	2.05
TB08	2.02	TB27	2.02
TB09	2.05	TB32	2.10
TB10	2.00	TB43	2.19
TB11	2.06	TB46	1.94
TB12	2.04	TB47	2.02
TB13	2.05	TB48	2.01
TB14	2.00	TB49	2.03
TB15	2.11	TC01	20.3
TB16	1.98	TC02	2.03
TB17	2.03	TC03	2.02
TB18	2.01	TC04	2.02
TB19	2.11	TC05	2.05
TB20	2.10	TC06	2.11