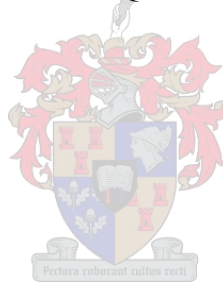


Ironing out Haemochromatosis: a study of an Indian family

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (MSc) in Genetics at the University of Stellenbosch.

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March 2008

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

Iron metabolism disorders comprise the most common disorders in humans. Hereditary haemochromatosis (HH) is a common condition resulting from inappropriate iron absorption. The most common form of the disease (Type 1) is associated with mutations in the *HFE* gene. The C282Y homozygous genotype accounts for approximately 80% of all reported cases of HH within the Caucasian population. A second *HFE* mutation, H63D, is associated with less severe disease expression. The C282Y mutation is extremely rare in Asian and African populations. The H63D mutation is more prevalent and has been observed in almost all populations.

Iron overload resulting from haemochromatosis is predicted to be rare in Asian Indian populations and is not associated with common *HFE* mutations that are responsible for HH in the Caucasian population. The aberrant genes associated with HH in India have not yet been identified.

The present study attempted to identify variants in six iron regulatory genes that were resulting in the Type 1 HH phenotype observed in two Asian Indian probands from a highly consanguineous family.

The promoter and coding regions of the *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* and *HJV* genes were subjected to mutation analysis. Gene fragments were amplified employing the polymerase chain reaction (PCR) and subsequently subjected to heteroduplex single-strand conformational polymorphism (HEX-SSCP) analysis. Samples displaying aberrations were then analysed using bi-directional semi-automated DNA sequencing analysis to identify any known or novel variants within the six genes. Variants disrupting restriction enzyme recognition sites were genotyped employing restriction fragment length polymorphism (RFLP) analysis.

Mutation analysis of the six genes revealed 24 previously identified variants, five novel variants (*HFE*: 5'UTR-840T→G, *CYBRD1*: 5'UTR-1813C→T, 5'UTR-1452T→C, 5'UTR-1272T→C; *HJV*: 5'UTR-534G→T, 5'UTR-530G→T), one previously described

microsatellite and two novel repeats. Variants identified within the *SLC40A1*, *CYBRD1* and *HJV* genes do not seem to be associated with the iron overload phenotype.

A previously described *HAMP* variant (5'UTR-335G→T) was observed in the homozygous state in both probands. This variant seems to be the genetic aberration responsible for iron overload in this Indian family. The severe juvenile haemochromatosis phenotype usually associated with *HAMP* mutations, was not exhibited by the two Indian probands. Their symptoms resembled those observed in classic Type 1 HH. It is suggested that variants identified in the *HMOX1* and *HFE* genes are modifying the effect of the *HAMP* variant and resulting in the less severe disease phenotype. Although this variant has only been identified in one Indian family, it could shed some light in the hunt for the iron-loading gene in India.

Opsomming

Oorerflike hemochromatose (OH) is 'n algemene siektetoestand wat ontstaan as gevolg van oneffektiewe opname van yster in die liggaam. Die mees algemene vorm van die siekte (Tipe 1) word geassosieer met mutasies in die *HFE*-geen. Die C282Y homosigotiese genotipe is verantwoordelik vir ongeveer 80% van alle gerapporteerde gevalle van OH binne die Kaukasiese bevolking. 'n Tweede *HFE* mutasie, H63D, word geassosieer met minder ernstige siekte simptome. Die C282Y mutasie is besonder skaars in Asiese en Afrika bevolkings.

Daar word bespiegel dat oorerflike ysteroorlading as gevolg van hemochromatose skaars is in Asiese Indiër bevolkings en word nie geassosieer met algemene *HFE* mutasies wat verantwoordelik is vir OH in Kaukasiese bevolkings nie. Die abnormale gene wat wél geassosieer word met OH in Indië is tot dusver nog nie identifiseer nie.

Die doel van hierdie studie was om die variante in ses yster-regulerende gene te identifiseer wat die Tipe 1 OH fenotipe in hierdie familie veroorsaak. Hierdie fenotipe is waargeneem in twee Asies Indiese familieledede afkomstig van 'n bloedverwante familie.

Die promotor en koderingsareas van die *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* en *HJV* gene is gesif vir mutasies. Geen fragmente is geamplifiseer met behulp van die polimerase kettingsreaksie (PKR) en daarna aan heterodupleks enkelstring konformasie polimorfisme (HEX-SSCP) analise blootgestel. PKR produkte wat variasies getoon het, is daarna geanaliseer deur tweerigting semi-geoutomatiseerde DNS volgorde-bepalingsanalise om enige bekende of nuwe variante binne die ses gene te identifiseer. Variante waar restriksie ensiem herkenningssetels teenwoordig is, is verder analiseer met behulp van die restriksie fragment lengte polimorfisme (RFLP) analise sisteem.

Mutasie analise van die ses gene het 24 bekende variante, vyf nuwe variante (***HFE***: 5'UTR-840T→G, ***CYBRD1***: 5'UTR-1813C→T, 5'UTR-1452T→C, 5'UTR-1272T→C, ***HJV***: 5'UTR-534G→T, 5'UTR-530G→T), een bekende herhaling en twee nuwe herhalings gewys. Variante wat binne die *SLC40A1*, *CYBRD1* en *HJV* gene geïdentifiseer is, blyk nie om by te dra tot die ysteroorladings-fenotipe nie.

Die bekende *HAMP* variant (5'UTR-335G→T) is waargeneem in die homosigotiese toestand in beide van die aangetaste individue. Hierdie variant blyk om die genetiese fout te wees wat verantwoordelik is vir die ysteroorlading in die betrokke Indiese familie. Die erge juveniele-hemochromatose fenotipe wat meestal geassosieer word met *HAMP*-mutasies, is nie waargeneem in hierdie familie nie. Hul simptome kom ooreen met die simptome van die klassieke Tipe 1 OH. Dit blyk moontlik te wees dat die variante identifiseer in die *HMOX1* en *HFE* gene die impak van die *HAMP* variant modifiseer en die matiger siekte-fenotipe tot gevolg het. Alhoewel hierdie variant slegs in een Indiese familie geïdentifiseer is, kan dit lig werp op die soektog na die veroorsakende ysterladingsgeen in Indië.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations are listed in alphabetical order.

2'	2-prime end
3'	3-prime end
5'	5-prime end
α	Alpha
β	Beta
$^{\circ}\text{C}$	Degrees Celsius
=	Equal to
γ	Gamma
>	Greater than
$\mu\text{g/ml}$	Microgram per millilitre
$\mu\text{g/l}$	Microgram per litre
μl	Microlitre
$\mu\text{mol/l}$	Micromole per litre
-	Minus
%	Percentage
+	Plus
\pm	Plus-minus
®	Registered trademark
<	Smaller than
\times	Times
A	Adenine residue
A (amino acid)	Alanine
A1	Allele 1
A2	Allele 2
A3	Allele 3
AA	Acrylamide
AIO	African iron overload
ALT	Alanine aminotransferase

AML1	Acute myeloid leukemia 1 protein
Ann	Annealing temperature
AP1	Activator protein 1
AP2	Activator protein 2
AP-2 α A	Activating enhancer-binding protein 2- α
AP-2 α B	Activating enhancer-binding protein 2- β
APS	Ammonium persulphate: (NH ₄)S ₂ O ₈
ASSP	Alternative Splice Site Predictor
AST	Aspartate aminotransferase
ATG	Translation initiation site
BAA	Bisacrylamide
Bach1	BTB and CNC homology 1
bp	Base pair
BMP	Bone morphogenetic protein
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin
C	Cytosine residue
C (amino acid)	Cysteine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
CEPH	<i>Centre d'Etude du Polymorphisme Humaine</i>
c-myb	Myb proto-oncogene protein
CNS	Central nervous system
CO	Carbon monoxide
Cont	Continued
COS-1 cells	African green monkey kidney cell line
CREB1	cAMP response element binding protein
CTCF	CCCTC binding factor
C-terminal	COOH terminal
<i>CYBRD1</i>	Cytochrome b reductase 1 gene

D (amino acid)	Aspartic acid
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
<i>DCYTB</i>	Duodenal cytochrome b gene
DCT1	Divalent cation transporter-1
ddH ₂ O	Double distilled water
del	Deletion
dGTP	2'-deoxy-guanosine-5'-triphosphate
dHPLC	Denaturing high-performance liquid chromatography
DMT1	Divalent metal transporter-1
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleotide
DTT	Dithiothreitol
dTTP	2'-deoxy-thymidine-5'-triphosphate
E (amino acid)	Glutamic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra-acetic acid: (C ₁₀ H ₁₆ N ₂ O ₈)
e.g.	For example
ELK1	ETS domain-containing protein ELK1
ER	Endoplasmic reticulum
ESE	Exonic splice element
<i>et al</i>	And others
EtBr	Ethidium bromide
EtOH	Ethanol
ETS	ETS oncogene
ETS1	Protein C-ets-1
ETS2	Protein C-ets-2
FD	Ferroportin disease
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
FISH	Fluorescence in situ hybridisation
FOXC1	Forkhead box protein C1

FOXL1	Forkhead box protein L1
FPN1	Ferroportin 1
g	Gram
G	Guanine residue
G (amino acid)	Glycine
GABP α	GA-binding protein α chain
GATA1	GATA-binding protein 2
g/l	Grams per litre
H (amino acid)	Histidine
H ⁺	Hydrogen ion
<i>HAMP</i>	Hepcidin antimicrobial peptide gene
H ₃ BO ₃	Boric acid
HCC	Hepatocellular carcinoma
HCP1	Haem carrier protein-1
HEPC	Hepcidin protein
Het	Heterozygous genotype
HEX-SSCP	Heteroduplex single-strand conformation polymorphism
<i>HFE</i>	High-iron gene
<i>HFE2</i>	Hemojuvelin gene
H-ferritin	Heavy chain ferritin
HH	Hereditary haemochromatosis
<i>HJV</i>	Hemojuvelin gene
HLA-A	Human leukocyte antigen A
HLA-H	Human leukocyte antigen-haemochromatosis
<i>HMOX1</i>	Haem oxygenase-1 gene
HNF	Hepatocyte nuclear factor
HNF4A	Hepatocyte nuclear factor 4- α
Hom	Homozygous variant allele
<i>HpyCH41V</i>	<i>Escherichia. coli</i> strain carrying the cloned <i>HpyCH41</i> gene from <i>Helicobacter pylori</i> CH4
I (amino acid)	Isoleucine

LIST OF ABBREVIATIONS AND SYMBOLS

IDT	Integrated DNA Technologies
i.e.	Latin phrase <i>id est</i> meaning “that is”
IKZF1	Ikaros family zinc finger 1
IL4	Interleukin-4
IL6	Interleukin-6
IL1- α	Interleukin-1 α
Inc	Incorporated
<i>In silico</i>	Refers to research conducted using computers only
<i>In vivo</i>	Latin phrase meaning “in body” or within a living organism
IRE	Iron-responsive element
IREG1	Iron-regulated transporter 1
IRP	Iron-regulatory protein
IVS	Intervening sequence
JH	Juvenile haemochromatosis (Type 2 haemochromatosis)
K (amino acid)	Lysine
KAc	Potassium acetate
kb	Kilobase
KCl	Potassium chloride
kD	Kilodalton
KHCO ₃	Potassium hydrogen carbonate
KH ₂ PO ₄	Potassium di-hydrogen orthophosphate
LEAP1	Liver-expressed antimicrobial peptide 1
LEF1	Lymphoid enhancer-binding factor 1
L-ferritin	Light chain ferritin
Ltd	Limited
Lys	Lysine
M (amino acid)	Methionine
<i>MaeII</i>	<i>Methanococcus aeolicus</i> strain 2
MAX	Myc-associated factor X
Met	Methionine

LIST OF ABBREVIATIONS AND SYMBOLS

mg	Milligram
MgAc	Magnesium acetate
MgCl ₂	Magnesium chloride
mg/kg	Milligram per kilogram
mg/ml	Milligram per millilitre
MHC	Major histocompatibility complex
ml	Millilitre
mM	Millimolar
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MTP1	Metal transporter protein-1
n	Number of
N (amino acid)	Asparagine
N	Homozygous wild type allele
NaCl	Sodium chloride
Na ₂ HPO ₄	Di-sodium hydrogen orthophosphate anhydrous
NEBuffer	New England Biolabs buffer
NF-1	Neurofibromin
NF-Y	Nuclear factor Y
ng	Nanogram
ng/ml	Nanograms per millilitre
(NH ₂) ₂ CO	Urea
NH ₄ Cl	Ammonium chloride
(NH ₄) ₂ SO ₄	Ammonium sulphate buffer
N/O	Not optimised
NR3C1	Nuclear receptor subfamily 3 group C member 1
NRAMP2	Natural resistance-associated macrophage protein 2
NTBI	Non-transferrin bound iron
OMIM TM	Online Mendelian Inheritance in Man TM
p	Short arm of chromosome
P	Phosphorous

P (amino acid)	Proline
PAA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA3	ETS domain-containing transcription factor PEA3
pH	Indicates acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
pmol	Picomole
Pro	Proline
PU.1	Spleen focus forming virus proviral integrating oncogene 1
PXR-1	Pregnane X receptor
q	Long arm of chromosome
Q (amino acid)	Glutamine
R (amino acid)	Arginine
RI	BMP Type I receptor
RII	BMP Type II receptor
RACE	Rapid amplification of cDNA ends
RAR- α 1	Retinoic acid receptor α 1
RAR- β	Retinoic acid receptor β
RAR- γ	Retinoic acid receptor γ
RFLP	Restriction fragment length polymorphism
RGM	Repulsive guidance molecule
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase PCR
RUNX2	Runt-related transcription factor 2
RXR- α	Retinoid X receptor α
RXR- β	Retinoid X receptor β
S (amino acid)	Serine
SDS	Sodium dodecyl sulphate

<i>SLC11A3</i>	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 gene
<i>SLC40A1</i>	Solute carrier family 40 (iron-regulated transporter) member 1 gene
SMAD3	Mothers against decapentaplegic homolog 3
SMAD4	Mothers against decapentaplegic homolog 4
SNP	Single nucleotide polymorphism
SOX9	SRY-related high-mobility group (HMG) box-9
SP1	Specificity protein 1
SP2	Specificity protein 2
SP3	Specificity protein 3
SP4	Specificity protein 4
SRY	Sex-determining region Y
SSCP	Single-strand conformation polymorphism
STAT1	Signal transducer and activator of transcription 1- α/β
STAT3	Signal transducer and activator of transcription 3
STEAP3	Six-transmembrane epithelial antigen of the prostate-3
SXR	Steroid and xenobiotic receptor
T	Thymine residue
T (amino acid)	Threonine
T3R	Thyroid hormone receptor
TA	Tris-acetate
TAA	Stop codon
TAG	Stop codon
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	Tris-Borate/EDTA buffer
TBI	Transferrin-bound iron
TEMED	N, N, N' N',-tetramethylethylenediamine: C ₆ H ₁₆ N ₂
TFBS	Transcription factor binding sites
TFR	Transferrin receptor
<i>TFR2</i>	Transferrin receptor 2 gene
TGA	Stop codon
T _m	Melting temperature

LIST OF ABBREVIATIONS AND SYMBOLS

™	Trademark
Tnf- α	Mouse tumour necrosis factor α
TNF- α	Human tumour necrosis factor α
Tris-HCl	Tris(hydroxymethyl)aminomethane [(CH ₂ OH) ₃ CNH ₂ -Cl]
TS	Transferrin saturation
TS%	Transferrin saturation percentage
<i>TspRI</i>	<i>Escherichia coli</i> strain carrying the cloned <i>TspRI</i> gene from <i>Thermus</i> species R
U	Units
U	Uracil residue
UK	United Kingdom
USA	United States of America
USF1	Upstream stimulatory factor 1
USF2	Upstream stimulatory factor 2
UTR	Untranslated region
v	Version
V (amino acid)	Valine
V	Volts
VDR	Vitamin D3 receptor
VNTR	Variable number tandem repeat
v/v	Volume per volume
WHO	World Health Organisation
w/v	Weight per volume
X	Termination or stop codon
Y (amino acid)	Tyrosine

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Dedicated to my family

‘Other things may change us, but we start and end with the family.’ Anthony Brandt

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following institutions and individuals without whom the completion of this thesis would have been impossible:

The National Research Foundation (Thuthuka), Molecular Research Foundation of South Africa and Stellenbosch University for providing financial support.

Stellenbosch University for providing the infrastructure and facilities necessary to complete this study.

My supervisor Dr Monique Zaahl for allowing me to carry out my research in her research laboratory, for her support, encouragement, patience and critical reading of various drafts of this thesis.

My co-supervisor Dr Richard Hift for supplying the samples analysed in this study and for critical reading of this thesis.

Prof RT Erasmus for performing biochemical iron analysis of patients' blood samples.

Natalie Strickland, Veronique Human and Nathan McGregor for their invaluable assistance in the lab.

Nicola Panton for her assistance and support throughout the writing of this thesis.

My family (Glynis Geere, Mark Geere and Tammy Hallendorff) for always being strong for me when it all seemed too much.

My friends for their support and encouraging words throughout the past two years.

To my heavenly Father for being my strength and allowing me to remember that 'This too shall pass.'

CHAPTER ONE

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 Introduction to Hereditary Haemochromatosis (HH)

Hereditary haemochromatosis (HH) (OMIMTM 235200) is a genetically and clinically heterogeneous condition that results from inappropriate dietary iron absorption. There have been great advances in the understanding of this condition since it was first described in 1865 as a “classic triad” of cirrhosis of the liver, diabetes mellitus and bronzing of the skin (reviewed by Limdi and Crampton, 2004). In 1889, von Recklinghausen coined the term haemochromatosis, describing a condition resulting from disrupted iron absorption and the resultant tissue damage. It was then Sheldon (1935) who explained the hereditary nature of the disease. Simon and colleagues (1976) demonstrated the close association between haemochromatosis and the major histocompatibility complex (MHC). Later they refined their findings and demonstrated that HH showed an association with the human leukocyte antigen (HLA)-A3 complex. Subsequently, haemochromatosis was linked to HLA-A on the short arm of chromosome 6 (Simon *et al*, 1976). Finally, in 1996, Feder and his colleagues identified the gene implicated in HH (Feder *et al*, 1996). The gene was initially named HLA-H for haemochromatosis but was then renamed *HFE* by the WHO Nomenclature Committee for Factors of the HLA system (Bodmer *et al*, 1997).

The identification of the *HFE* gene and the causative variants in this gene has greatly improved the understanding of the HH condition. Feder and his colleagues (1996) identified the C282Y variant in the *HFE* gene and found that the vast majority of HH patients were homozygous for this variant. In patients homozygous for the C282Y variant, the iron overload phenotype is variable. Basset and his colleagues (1986) noted that iron stores differed by as much as ten-fold amongst homozygous individuals (reviewed by Bomford, 2002). Although not fully understood, environmental factors or genetic modifiers of the C282Y variant can partly explain this anomaly. The extent to which individuals are affected seems to depend on the severity of the genetic defect, age, sex, environmental stimuli such as dietary iron intake, the extent of iron loss due to other processes such as blood donation, and the presence of other diseases or toxins e.g. Hepatitis C virus, excess ethanol intake, and porphyria cutanea tarda (Bothwell and MacPhail, 1998; Chapman *et al*, 1982; di Bisceglie *et al*, 1992). The type and amount of iron that individuals consume could influence phenotype but because most HH

patients do not take iron supplements, this does not seem to be an important modifier. The effect of alcoholism on the HH phenotype has always been recognised by researchers (reviewed by Fletcher and Powell, 2003). In 1935, Sheldon noted that one fifth of all HH patients had a history of alcoholism (reviewed by Beutler, 2003). The effect of alcohol on the HH phenotype is not clear though because it has been reported that some non-drinkers and people who hardly ever consume alcohol are affected to the same degree as more regular alcohol consumers. This may indicate that alcohol is a secondary factor and not the only or most important modulator of C282Y expression.

Several groups have tried to explain the role that genetic modifiers play in the variability of the HH phenotype. The hepcidin antimicrobial peptide (*HAMP*) gene is of particular interest as it has been found to modulate the phenotype of the C282Y variant in mice. Nicolas and colleagues (2004) intercrossed *Hfe*-knockout mice (*Hfe*^{-/-}) with mice with one normal *HAMP* gene (*Usp2*^{+/-}). They noted that liver iron accumulation was more severe in the *Hfe*^{-/-}*Usp2*^{+/-} mice than in the *Hfe*^{-/-} mice. They therefore concluded that haploinsufficiency of hepcidin does intensify the HH phenotype and provides a genetic explanation for the phenotypic variability of HH. Jacolot and colleagues (2004) performed similar experiments and supported these conclusions when they identified *HAMP* variants in the heterozygous state in five patients who were also homozygous for the *HFE* C282Y variant. These variants included one that replaced arginine with glycine at amino acid position 59 (R59G), a second that replaced glycine with aspartic acid at amino acid position 71 (G71D) and a third that created a premature stop codon at amino acid position 56 (R56X). The iron indices of these five patients were among the most elevated of the study cohort. Based on these observations they concluded that variants in the *HAMP* gene could exacerbate the phenotypic expression of the C282Y homozygous phenotype. While screening the same study cohort as Jacolot *et al* (2004), Le Gac and colleagues (2004a) identified nine C282Y homozygotes who were also heterozygous for missense mutations in the hemojuvelin (*HJV*) gene. These nine individuals had significantly higher mean serum ferritin (SF) levels and thus *HJV* is implicated as another modifier of HH expression. Hofmann *et al* (2002) performed mutation analysis on the transferrin receptor 2 (*TFR2*) gene in two male siblings who were homozygous for the C282Y variant but whose phenotypes differed. They identified a variant within the *TFR2* gene in the brother with liver fibrosis and concluded that *TFR2* could function as a modifier for the penetrance of the HH phenotype when inherited in conjunction with the C282Y homozygous genotype. Although a great deal of progress has been made, further research is necessary to

identify other genes that may modify the HH phenotype and in part, explain the phenotypic heterogeneity and incomplete penetrance of this condition.

After the identification of the *HFE* gene (Feder *et al*, 1996) it was found that not all haemochromatosis patients were carriers of *HFE* variants and this led to the discovery of other forms of haemochromatosis. Classic or Type 1 HH is the most common form which is inherited in an autosomal recessive manner and is associated with variants in the *HFE* gene. The second and more severe type of HH is juvenile haemochromatosis (JH) or Type 2 HH. JH is also an autosomal recessive disorder and is associated with aberrations in the *HJV* (Type 2A) and the *HAMP* genes (Type 2B). Variants in the *TFR2* gene are responsible for Type 3 HH, which is also inherited in an autosomal recessive manner. Type 4 HH or the ferroportin disease is inherited in an autosomal dominant manner and results from variants in the solute carrier family 40 (iron-regulated transporter) member 1 (*SLC40A1*) gene. The second autosomal dominant form of HH is Type 5 HH. This disorder has only been identified in one family and is associated with aberrations in the H-ferritin gene. Another condition resulting from iron overload has been denoted African iron overload and affects people of African descent but the causative gene has yet to be identified. All of these disorders result from aberrations that alter iron metabolism and/or homeostasis, which leads to iron overload and they will be discussed further.

1.1.1 *HFE*-associated HH (Classic or Type 1)

1.1.1.1 Pathophysiology

Type 1 HH (OMIMTM 235200) is an autosomal recessive condition that affects approximately 1 in every 100 South Africans of northern European descent (Meyer *et al*, 1987; de Villiers *et al*, 1999) and approximately 1 of every 200 Caucasian individuals of northern European descent worldwide (Merryweather-Clarke *et al*, 1997). The disruption in iron absorption in HH patients leads to iron overload and the excess iron is deposited in tissues such as the liver, heart, pancreas, joints and pituitary gland (Witte *et al*, 1996). Iron is deposited in the hepatocytes with a decreasing gradient from periportal zone to centrilobular area and although typical is not unique to HH, but relative sparing of Kupffer cells is typical of HH and not seen in individuals with secondary iron overload.

Iron can readily exchange electrons in aerobic conditions and is thus essential for basic cellular functions such as cellular respiration, DNA synthesis, and oxygen transport. The excess iron in the tissues of HH patients can, however, be hazardous as it catalyses the conversion of hydrogen peroxide to free radical oxygen species that attack cell membranes, proteins and DNA (Andrews, 1999).

Iron overload occurs relatively slowly in *HFE*-associated haemochromatosis. By the fourth decade of life, patients show no symptoms but have accumulated 10 to 20 grams of iron in their parenchymal tissues. In men clinical expression of HH usually presents at 40 to 60 years of age. Due to the regular loss of iron through menstruation, pregnancy and lactation, iron overload is delayed by approximately one decade in women. The symptoms of female patients usually become evident only after menopause. This may explain why 2-10 times more men are afflicted by HH than women (Moirand *et al*, 1997).

The rate at which iron accumulates in the tissues and the severity of clinical symptoms differ noticeably in each patient. Early symptoms include unexplained fatigue, weakness, joint pain, heart palpitations, weight loss, loss of libido, depression and abdominal pain (Adams *et al*, 1997). Because these symptoms are indefinite, HH can go undiagnosed at this stage. When the condition proceeds untreated it may result in more severe symptoms such as liver cirrhosis, arthritis, skin hyperpigmentation, diabetes mellitus, hypopituitarism, hypogonadism, chronic abdominal pain, cardiomyopathy, primary liver cancer or an increased risk of infection by certain bacteria (Adams *et al*, 1997).

1.1.1.2 Genetic mutations associated with Classic HH

In the study by Feder *et al* (1996) two missense mutations were initially identified in patients with HH. The first resulted in a single base transition resulting in a change from cysteine at amino acid position 282 to tyrosine (C282Y) and the second was a change of histidine to aspartate at amino acid position 63 (H63D) of the gene. Of the 178 HH patients studied, 148 (83%) were homozygous for the C282Y mutation and 8 (4%) were compound heterozygotes for the C282Y and the H63D mutation. A third variant in *HFE* replaces the amino acid serine with cysteine (S65C) and is present in approximately 1.5% of European individuals (Mura *et al*, 1999; Beutler *et al*, 2000). At first described as a polymorphism, the S65C/C282Y genotype may predispose individuals to a milder form of HH. Other variants have been

identified in the *HFE* gene in individuals with HH, including one that results in the replacement of isoleucine at amino acid position 105 with threonine (I105T) and another that results in the replacement of glycine with arginine at amino acid position 93 (G93R) which were identified in two families from Alabama (Barton *et al*, 1999). Two other variants were identified in the Italian population including one that causes glycine to be replaced with threonine at amino acid position 168 (G168T) and the second where alanine replaces glycine at amino acid position 169 (G169A) (Piperno *et al*, 2000). How these variants disrupt iron homeostasis still needs to be elucidated. Variants in the *HFE* gene are often inherited together with the C282Y heterozygous or homozygous genotype or with the H63D variant.

The common *HFE* mutation, C282Y, disrupts an S-S bond in the $\alpha 3$ domain of the protein. This domain is essential for the noncovalent interaction between HFE and $\beta 2$ -microglobulin and the C282Y variant abolishes this interaction leading to decreased presentation of HFE on the cell surface (Waheed *et al*, 1997). The role of H63D is still not certain but interestingly, it seems to form a salt bridge with a residue in the $\alpha 2$ domain that binds HFE to transferrin receptor 1. When HFE is bound to $\beta 2$ -microglobulin it forms an association with TFR1 in the duodenal precursor cell membrane and assists in the transport of transferrin-bound iron into these cells. Disruption of this function could result in increased iron absorption from the duodenal lumen.

The C282Y homozygous genotype results in the most severe form of Type 1 HH followed by the C282Y/H63D and H63D/H63D phenotypes. Although approximately five of every 1000 individuals is homozygous for the C282Y variant, this variant seems to display incomplete penetrance. The proportion of HH patients homozygous for the C282Y variant differs in different populations; it ranges from approximately 64% in an Italian study (Carella *et al*, 1997) to 100% in an Australian study (Jazwinska *et al*, 1995) and is absent from the Asian and African populations (Merryweather-Clarke *et al*, 1997; Roth *et al*, 1997). Although most individuals of northern European descent presenting with clinical symptoms of HH are homozygous for the C282Y variant, determination of the C282Y allele frequency has shown a large discrepancy between the number of C282Y homozygotes and the number of patients diagnosed with HH (Bomford, 2002). Also, individuals in the general population have been found to be homozygous for the C282Y variant and do not exhibit HH symptoms (reviewed by Adams, 2000). Thus the C282Y variant exhibits incomplete penetrance. Environmental

and genetic modifiers of the HH phenotype could explain why not all C282Y individuals are affected and not all homozygotes are affected to the same extent.

Merryweather-Clarke *et al* (2003) reported digenic inheritance of HH in two families. One proband in the first family was heterozygous for the C282Y variant as well as a four base pair deletion in the *HAMP* gene that removes the last nucleotide of exon 2 that encodes methionine and the first nucleotide of intron 2 [Met50del IVS2+1(-G)]. The proband exhibited a severe form of HH similar to juvenile haemochromatosis. The Met50del IVS2+1(-G) variant was absent from 321 control subjects. This variant disrupts the GT splicing acceptor site of the gene and produces a different open reading frame in exon 3. In the second family a less severe *HAMP* variant was identified (G71D). This variant was present in the control cohort and different ethnic groups. This variant could interfere in correct protein folding. Although true digenic inheritance is rare in HH, they suggest that mechanisms including digenic inheritance could be playing a role in the pathophysiology of HH and could explain the heterogeneity of the HH phenotype. The authors suggest that heterozygosity for *HAMP* variants, which disrupt its function in iron homeostasis, could modulate the phenotype of individuals heterozygous or homozygous for the C282Y variant in *HFE*. They also postulate that the severity of the *HAMP* variant will influence the severity of the iron overload phenotype. Therefore the C282Y variant is a necessary, but not the sole causative factor for the development of clinical symptoms of HH.

1.1.1.3 Diagnosing Classic HH

Although the discovery of the *HFE* gene has greatly modified diagnostic and screening approaches, one must remember that C282Y homozygosity alone does not necessarily mean that haemochromatosis will develop. The HH phenotype is determined by genetic, biochemical and clinical factors but there is no agreement between clinicians which factor or combination of factors defines HH. Diagnosis of HH is complicated by the variability of the HH phenotype but various tests have been developed to assist in the diagnostic process. These include biochemical (serum iron studies), genetic testing and liver biopsy (reviewed by Pietrangelo *et al*, 2003). Another method for assessing iron overload is by measuring the number of phlebotomies required to regain normal serum iron and ferritin levels. The most common biochemical tests used to assess body iron status are transferrin saturation percentage (TS%) ($TS\% = \text{serum iron} / \text{total iron binding capacity} \times 100$) and SF levels. In HH, iron

initially accumulates in the transferrin pool, which results in an increase in transferrin saturation (TS), and subsequently in the tissue parenchyma. As the iron is accrued in the parenchyma there is an accompanying increase in SF concentration. TS is usually elevated prior to symptom manifestation so it is an early indicator of the HH condition (Hanson *et al*, 2001). TS cutoff values vary from 45-70% but it has been reported that values of 60% or more in men and 50% or more in women have an estimated sensitivity of 92%, specificity of 93% and positive predictive value of 86% for detecting C282Y homozygous individuals with HH (Tavill, 2001). If TS is elevated and no other explanation for iron overload exists (e.g. chronic anaemias, liver diseases due to excessive alcohol consumption or viral infection), it may indicate that the individual has HH. Ferritin is an iron storage protein and SF concentration is a good estimate of total body iron stores (1 ng/ml = 10 mg stored iron) (McDonnell and Witte, 1997). SF levels, but not TS, are associated with clinical signs of HH and are higher for individuals with clinical manifestations of HH (Bradley *et al*, 1996). Ferritin values exceeding 200 µg/l in premenopausal women and 300 µg/l in men and postmenopausal women are suggestive of HH (Burke *et al*, 1998). If TS as well as SF levels are elevated, additional diagnostic testing, such as liver biopsy or quantitative phlebotomy, should be performed to verify that iron overload is present.

The discovery of the *HFE* gene (Feder *et al*, 1996) has greatly altered the approach for diagnosing HH. Genetic tests are readily available and genotyping can confirm HH. In patients where HH is highly suspected C282Y and H63D mutation analysis should be performed. Mutation detection is especially important in individuals who do not carry the C282Y or H63D mutations. Pedigree analysis can be performed to identify if other variants in the *HFE* gene are playing a role or if other genes are involved in the clinical expression of the condition. In these families, TS and SF concentrations are used to screen for the HH phenotype. As described previously, the C282Y genotype does not confer the HH phenotype in all individuals. Genotype results should be considered together with clinical and biochemical results when diagnosing HH, as the clinical expression of the condition is widely variable. A combined genotype/phenotype approach would assist in the identification of modifying environmental and/or genetic factors that could contribute to or be protecting against the HH phenotype in individuals with atypical haemochromatosis (Lyon and Frank, 2001). As HH is a treatable genetic disorder, early diagnosis and treatment is essential to prevent organ damage, improve quality of life and longevity.

1.1.2 Juvenile Haemochromatosis (Type 2)

1.1.2.1 Pathophysiology

Juvenile or Type 2 haemochromatosis (JH) (OMIMTM 602390) is an autosomal recessive iron overload disorder. It is characterised by early onset iron overload that results in severe organ damage before the age of 30 years (Camaschella *et al*, 2002). Unlike *HFE* associated HH, males and females are both affected equally by JH. Increased TS% and SF levels are observed early in life in both sexes (reflecting plasma iron loading and excess tissue iron, respectively) (Cazzola *et al*, 1998). There is a daily increase in iron absorption, which surpasses that of *HFE* haemochromatosis, and iron accumulation occurs at a more rapid rate in JH (Lamon *et al*, 1979). Excess iron is deposited in the parenchymal cells in a similar manner as seen in *HFE*- and *TFR2*-haemochromatosis or Type 3 (See Section 1.1.3).

Symptoms of JH are similar to those of *HFE* haemochromatosis. A combination of cardiac disease, liver cirrhosis, hypogonadism, diabetes, arthropathies and skin pigmentation may result but are more severe than in *HFE* type. Cardiac involvement and hypogonadism are the characteristic features of JH and are more frequent than liver disorders. This could be a reflection of the different susceptibilities of the cells to massive iron overload during organ development (Lamon *et al*, 1979). If the disease goes untreated, cardiac symptoms will govern the course of the disease with heart failure and/or major arrhythmias being the leading cause of death (Camaschella *et al*, 2002; De Gobbi *et al*, 2002).

1.1.2.2 Genetic mutations associated with JH

The early onset and severity of iron overload in JH as well as the equal penetrance in both sexes implies that the aberrant protein responsible for JH must play a more important role in the inhibition of iron absorption than *HFE* and *TFR2* (De Gobbi *et al*, 2002). This prediction was confirmed by the discovery of the JH gene, hepcidin antimicrobial peptide (*HAMP*) (Roetto *et al*, 2003) and subsequent identification of mutations within this gene associated with the disease (Roetto *et al*, 2004; Matthes *et al*, 2004).

Another gene, termed *HFE2* or hemojuvelin (*HJV*) has been identified with amino acid substitution 320 G→V accounting for two-thirds of the mutations identified (Papanikolaou *et*

al, 2004). The *HJV* gene is implicated in the most frequent form of JH on the basis of the discovery of six variants, found in either the homozygous or compound heterozygous state. These variants were identified in 12 unrelated families from Greek, Canadian and French descent (Papanikolaou *et al*, 2004). Eighteen other *HJV* variants have been identified in 31 families from England, Albania, Italy, Southeast USA, Australia, France and Saguenay-Lac-Saint Jean (Quebec) (Papanikolaou *et al*, 2004; Lanzara *et al*, 2004; Lee *et al*, 2004; Huang *et al*, 2004). The 320 G→V variant was found in 34 of the 60 patients (56.7%) but all the other variants were identified in single families. The majority of these variants generate premature stop codons or are missense substitutions affecting conserved amino acid residues.

HJV and *HAMP* inactivation cause the same disease and it is impossible to predict mutations in either protein from clinical manifestations (Lanzara *et al*, 2004). Although the function of *HJV* is not well defined it has been reported that in patients with *HJV* mutations and in *HJV* knockout mice (*Hfe2^{-/-}*), hepcidin levels are extremely low. This could signify that *HJV* and hepcidin function in the same pathways and that *HJV* positively modulates hepcidin expression (Papanikolaou *et al*, 2004). Babitt *et al* (2006) reported that *HJV* regulation of hepcidin occurs through the bone morphogenetic protein (BMP) signalling pathway where it acts as a coreceptor. The authors showed that BMP up-regulates hepcidin expression within hepatocytes and this process is enhanced in the presence of *HJV*. *HJV* mutations that cause JH were investigated to determine whether they had an effect on BMP signalling. It was observed that these mutations result in impaired BMP signalling ability and a decrease in hepcidin expression. These findings indicate that rather than JH being the result of two different and independent mechanisms, the underlying cause is a decrease in hepcidin expression, which results in aberrant iron regulation.

1.1.2.3 Diagnosing JH

For young adults with signs of JH the biochemical status is identical to those in individuals with Type 1 HH. Genetic testing in these patients will however require sequencing of the *HAMP* and *HJV* genes. Since these tests are not widely available, diagnosis may be based on liver biopsy specimens (Pietrangelo, 2004a). If an individual is diagnosed with JH then family members should undergo biochemical testing. If the causative mutation has been identified in the proband, then family members should also be referred for genetic testing as early detection and treatment could prevent the progression of the disease.

1.1.3 *TFR2*-associated Haemochromatosis (Type 3)

1.1.3.1 Pathophysiology

Type 3 haemochromatosis (OMIMTM 604250) was first identified in Southern Italy where there are very few haemochromatosis patients who are homozygous for the C282Y mutation in the *HFE* gene. Genome screening of affected families led to the identification of Type 3 haemochromatosis where patients presented with aberrations in the transferrin receptor 2 (*TFR2*) gene (Camaschella *et al*, 2000). Type 3 haemochromatosis displays autosomal recessive inheritance.

Although very few cases have been reported, the clinical phenotype resulting from variants in the *TFR2* gene are similar to those in *HFE* haemochromatosis. Increased serum iron parameters (TS% and SF) due to increased iron absorption at the duodenal level leads to parenchymal iron overload. Type 3 haemochromatosis predominantly affects the liver where iron is deposited in a periportal distribution. Iron loading due to *TFR2* inactivation occurs early in life, similar to JH, but the clinical manifestations of the disease are not as severe and vary according to the specific *TFR2* mutation (reviewed by Robson *et al*, 2004).

Type 3 haemochromatosis is very rare and is usually observed in families from the Central Southern parts of Italy although there are some exceptions. The causative *TFR2* variants are usually only found in the family in which they were identified (Roetto *et al*, 2002a).

1.1.3.2 Genetic mutations associated with Type 3 HH

Camaschella *et al* (2000) identified the first variant in the *TFR2* gene associated with Type 3 haemochromatosis. Several members of two Sicilian families were homozygous for a nonsense mutation that replaced tyrosine with a stop codon at amino acid position 250 (Y250X). It has never been detected in the heterozygous state in screening studies of Italian blood donors or in other studies worldwide (Roetto and Camaschella, 2005). The Y250X variant was identified in two young males, 3 and 16 years old, from the same geographical region as the original families. They presented with elevated TS and SF and had high hepatic iron indices (Piperno *et al*, 2004).

The AVAQ motif deletion, (AVAQ594-597del), was identified in three Italian siblings (Girelli *et al*, 2002) but unexpectedly, also in a Japanese family (Hattori *et al*, 2003). The Japanese individuals were older at diagnosis, had hepatic iron loading and liver cirrhosis was observed in one middle-aged man. The finding of the same deletion in two different ethnic groups causing similar phenotypic expression indicates that the AVAQ motif of the TFR2 protein may play an important role in iron regulation (Roetto and Camaschella, 2005).

Other variants in the *TFR2* gene have been reported including E60X (Roetto *et al*, 2001), M172K (Roetto *et al*, 2001), R455Q (Hofmann *et al*, 2002), Q690P (Mattman *et al*, 2002), V22I (Biasiotto *et al*, 2003) and R105X (Le Gac *et al*, 2004b), Q317X (Pietrangelo *et al*, 2005). The *TFR2* gene codes for two alternatively spliced forms, α and β (Kawabata *et al*, 1999). Most variants affect both isoforms, but some such as E60X and R105X only affect the α -form. The M172K variant in the *TFR2* gene has been associated with the most severe phenotype observed. This variant disrupts a methionine residue in the α -form, which is also the putative start site of the β -form. It has been reported that if at least one isoform remains intact, a less severe phenotype is observed. This was the case in patients with the E60X genotype: of the five patients studied, one female did not express the phenotype and one was iron deficient. However in young patients with the Y250X or the AVAQ deletion, both of which disrupt both TFR2 isoforms, it was reported that iron overload was severe and that two twenty-year-old patients had hypogonadism (Roetto and Camaschella, 2005).

Results from family screening studies have shown that individuals heterozygous for the *TFR2* variants described do not display the iron overload phenotype, even when in the compound heterozygous state with H63D *HFE* mutation (Roetto and Camaschella, 2005).

1.1.3.3 Diagnosing Type 3 HH

When unexplained iron overload is present in an individual and Type 3 HH is suspected, diagnosis must be confirmed through a process of elimination. This is because many of the symptoms of Type 3 HH mimic those of Type 1. In both disorders, symptoms usually manifest after the age of 30 years and the biochemical status is the same in both. Biochemical tests should initially be performed to determine if TS and SF levels are elevated. If these results are inconclusive, liver biopsy will confirm the presence of iron overload if it is present. If this is the case, genotyping for the common *HFE* variants, C282Y and H63D, must be

performed. If no *HFE* variants are present, mutation screening of the *TFR2* gene should be performed to confirm the diagnosis of Type 3 haemochromatosis.

1.1.4 *SLC40A1*-associated HH (Type 4)

1.1.4.1 Pathophysiology

Type 4 haemochromatosis is also referred to as the ferroportin disease (FD) (OMIM™ 606069), as it is associated with aberrations in the solute carrier family 40 (iron-regulated transporter) member 1 (*SLC40A1*) gene. This gene, also known as the solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 (*SLC11A3*) gene, ferroportin 1 (*FPN1*) gene, iron-regulated transporter 1 (*IREG1*) gene and metal transporter protein-1 (*MTP1*) gene, encodes the SLC40A1 or ferroportin protein. The first description of Type 4 haemochromatosis was described in two almost identical studies in the Netherlands (Njajou *et al*, 2001) and Italy (Montosi *et al*, 2001). This disease displays clinical and genetic features distinct from any of the other forms of haemochromatosis. FD is inherited in an autosomal dominant manner and results from heterozygous variants in the *SLC40A1* gene.

Most patients with FD present with elevated SF in the first decade of life and normal to low TS levels, which gradually increase in the third to fourth decades of life. Iron accumulation is progressive and iron is deposited mainly in the liver macrophages (Kupffer cells) and reticuloendothelial cells of young patients. Iron may become deposited in the hepatocytes of older patients. The biochemical penetrance of FD seems to be complete as all reported individuals with *SLC40A1* variants have increased SF levels regardless of the position of the variant in the mature protein (Pietrangelo, 2004b). In some FD cases individuals present with mild iron-deficient anaemia.

The clinical course of FD seems to be less severe than Type 1 haemochromatosis. It has been hypothesised that nonparenchymal cell (Kupffer cell) iron overload is better tolerated than parenchymal cell iron overload and is less fibrogenic (Gualdi *et al*, 1994). This could explain why FD does not progress into cirrhosis of the liver but is limited to the development of fibrosis (Gualdi *et al*, 1994) even when iron levels are extremely high. Although most patients have iron loading in the Kupffer cells, some studies have reported iron loading in hepatocytes

(Wallace *et al*, 2002). Iron is distributed in the liver in a homogenous lobular manner rather than the periportal and central distribution characteristic of Type 1 haemochromatosis.

Therapeutic phlebotomy is an effective iron depletion therapy but not all patients can endure weekly phlebotomies and may develop anaemia. SF levels remain elevated even after slight anaemia has been induced and TS levels are decreased. In these cases erythropoietin therapy may modify the effects of phlebotomy and be more beneficial to the patient. Defective iron export from the macrophages may be responsible for inadequate iron supply to erythroid precursors in the bone marrow, leading to latent anaemia and reduced tolerance to iron depletion. Defective iron export from macrophages, which in turn could be responsible for inadequate iron supply to erythroid precursors in the bone marrow could result in anaemia and an intolerance to iron depletion therapy.

1.1.4.2 Genetic mutations associated with Type 4 HH

The two original studies identified an atypical form of haemochromatosis that was not linked to *HFE* (Njajou *et al*, 2001; Montosi *et al*, 2001). A genome-wide search in both pedigrees showed linkage to markers on 2q32. The *SLC40A1* gene was later identified and it was reported that the affected Dutch and Italian family members were heterozygous for the N144H and A77D variants, respectively.

Since its original discovery, other variants in the *SLC40A1* gene have been described in patients with FD including V162del (Devalia *et al*, 2002; Cazzola *et al*, 2002; Roetto *et al*, 2002b; Wallace *et al*, 2002), D157G, Q182H, G323V (Hetet *et al*, 2003), N144T (Arden *et al*, 2003), Y64N (Rivard *et al*, 2003), Q248H (Gordeuk *et al*, 2003), G490D (Jouanelle *et al*, 2003), G80S, N174I (Pietrangelo, 2004b), N144D, C326Y (Robson *et al*, 2004), D270V (Zaahl *et al*, 2004), G80V, D181V, G267D (Cremonesi *et al*, 2005), C326S (Sham *et al*, 2005), N185D (Morris *et al*, 2005) R88T, and I180T (Bach *et al*, 2006). The vast majority of variants have been reported in single families but the V162del mutation has been reported in different families with different ethnicities. Although the condition is rare, *SLC40A1* variants have been described in people worldwide including families from the United Kingdom, Australia, Italy, Greece and African Americans.

The structure of the ferroportin protein is still unclear. Researchers have reported that the protein has 12 transmembrane domains (Liu *et al*, 2005) and that most of the identified variants are localised in the cytosolic regions of the ferroportin protein. They report that *SLC40A1* variants may be divided into two classes: variants that have a gain in function and those that result in a loss of function. Variants that result in a gain of function retain the ability to activate the iron-response proteins (IRPs) and iron is exported from the cells and ferritin is depleted. Loss of function variants inhibit IRP activation activity and cause only a slight decrease in SF levels.

It has been reported that when iron levels are high in the cells, hepcidin binds to ferroportin, internalising it in lysosomes within the cell and then degrades these lysosomes. This inhibits iron export from the cells (Nemeth *et al*, 2004a). In a study by Papanikolaou *et al* (2005) increased hepcidin levels were observed in patients with the V162del mutation. This may indicate a loss of responsiveness to hepcidin regulation leading to excess iron deposition in the tissues. Most of the *SLC40A1* variants studied by Liu *et al* (2005) occur in the cytosolic regions of the protein and it is unlikely that these cytosolic regions comprise the hepcidin binding site. They hypothesise that these variants may cause a conformational change in the ferroportin protein preventing hepcidin-mediated internalisation or organisation into lysosomes.

The clinical manifestations of FD are highly variable and there are various mechanisms that lead to the abnormal functioning of ferroportin. As clinical data accumulates a clearer understanding of the effects of *SLC40A1* variants on iron metabolism will develop.

1.1.4.3 Diagnosing Type 4 HH

As is the case with Type 1 and Type 3 HH, symptoms of Type 4 HH manifest after the age of 30. Initially, biochemical analysis should be performed to determine the patient's iron parameters. In contrast with Type 1 and 3 HH, SF levels are usually elevated prior to the increase in TS. Therefore, elevated SF along with normal to low TS (sometimes with mild anaemia) is indicative of Type 4 HH. To confirm the Type 4 HH diagnosis, mutation screening of the *SLC40A1* gene must be performed.

1.1.5 H-ferritin-associated HH (Type 5)

1.1.5.1 Pathophysiology

In 2001, a second form of autosomal dominant haemochromatosis was identified in a Japanese family (Kato *et al*, 2001). The proband, a 56-year-old female, had elevated SF and TS levels. Magnetic resonance imaging (MRI) was performed and low signal intensity, which is an indication of iron deposition, was identified in the liver, heart and bone marrow. A liver biopsy specimen showed heavy iron deposition in most of the hepatocytes as well as less iron deposition in Kupffer cells. Staining of a spleen specimen showed iron deposits in macrophages. Seven family members across three generations were studied and elevated SF levels were observed in three individuals. The proband's brother (aged 65) also presented with iron deposits in his liver and bone marrow.

1.1.5.2 Genetic mutations associated with Type 5 HH

The clinical manifestations in the family hinted at a form of hereditary haemochromatosis and all the individuals were screened for the *HFE* C282Y and H63D variants as well as the Y250X variant in the *TFR2* gene. These variants were not found in any of the family members. Further analysis was performed on the H- and L-ferritin genes by sequencing analysis. A single base pair conversion resulting in the replacement of alanine with threonine at amino acid position 49 (A49T) was identified in the second residue of the five base pair iron-responsive element (IRE) sequence of the H-ferritin mRNA. This variant was identified in the heterozygous state in four of the family members but only three of them had elevated SF levels. The fourth individual was the 28-year-old daughter of the proband and she had just given birth and was breastfeeding. These factors could have resulted in the lack of the iron overload phenotype due to an increased level of iron loss.

IRPs have been shown to interact with IREs (Haile *et al*, 1989) and influence protein expression. Functional analysis of the mutated mRNA demonstrated that the mutated IRE binds to the IRP with a higher binding affinity than the wild-type form. This indicates that the mutated IRE binds to the IRP strongly and thus inhibits the translation of H-subunit mRNA (Kato *et al*, 2001). Further analysis demonstrated that in the liver, expression of the H-subunit was suppressed while that of the L-subunit was elevated in comparison to the wild type form.

With regard to the tissue deposition, it is known that the H-subunit of ferritin performs a ferroxidase function to incorporate iron into the ferritin molecule (Harrison and Arosio, 1996). The researchers found that in the presence of the mutated H-subunit, iron incorporation into ferritin was much lower in transfected COS-1 cells compared to the wild type and that total cellular iron uptake was also higher. The researchers concluded that the increase in iron uptake resulted in more iron in the cytosol due to the loss of ferroxidase activity in the H-subunit.

Although this form of autosomal dominant haemochromatosis has only been identified in a single family, further research is necessary to determine if the variant in the H-ferritin gene is an isolated or a common one.

1.1.5.3 Diagnosing Type 5 HH

As mentioned, Type 5 HH has only been identified in a single Japanese family. Therefore, a molecular diagnostic test unique to this type of HH has not yet been developed. In this family, symptoms manifested after the age of 30 as is seen in Type 1, Type 3 and Type 4 haemochromatosis. The biochemical status of the proband was the same as is expected in Type 1 HH. Iron deposits were reported in hepatocytes as well as in macrophages, making it unique from Type 1 HH. More research is necessary, but liver biopsy may be a more definitive test for Type 5 haemochromatosis, as histological results will identify sites of iron deposition that differ from the other types of HH and may be unique to Type 5. As it now stands, mutational screening of the *HFE*, *TFR2* and *SLC40A1* genes will have to be performed initially, to determine if these are the causative genes. If they are eliminated as candidate genes, the H-ferritin gene must be screened to confirm the Type 5 HH diagnosis.

1.1.6 African Iron Overload (AIO)

1.1.6.1 Pathophysiology

Strachan (1929) first identified iron overload in sub-Saharan Africans. He studied 876 individuals from central and Southern Africa who had died in Johannesburg between 1925 and 1928. He concluded that iron overload was a common disorder affecting Africans and that the main cause of iron overload was their diet (Walker and Segal, 1999). For many years,

after it was first identified, it was believed that AIO was caused by excess iron intake from a home-brewed traditional beer, which is made in non-galvanised steel pots or drums (Bothwell *et al*, 1964). It is not known what the prevalence of AIO is in urban African populations but Gordeuk *et al* (1992a) estimated that approximately 10% or more of rural populations were affected.

Patients with AIO have elevated SF levels and to a lesser extent, TS levels. Iron deposits have been reported in the liver, heart, spleen, bone marrow, pancreas and kidneys of affected individuals. Not unlike Type 4 HH, iron is mostly deposited in the macrophages but has also been found in parenchymal cells of the various tissues. Many patients suffer from siderosis, fibrosis, and cirrhosis of the liver and there may be an aetiological association with hepatocellular carcinoma (HCC), tuberculosis (Moyo *et al*, 1997a) and other infections. There have also been patients identified with diabetes mellitus and osteoporosis. Because of the variable AIO phenotype clinicians often misdiagnose individuals with AIO.

1.1.6.2 Genetic mutations associated with AIO

The observation that not all beer drinkers developed iron overload led to the belief that a genetic factor was playing a role in the aetiology of the condition. Researchers have studied sub-Saharan and African-American populations with iron overload but neither of these populations shows linkage to the *HFE* gene (Gordeuk *et al*, 1992a; Barton *et al*, 1995). Gordeuk *et al* (1992b) set out to determine if a genetic factor, other than *HFE*, played a role in AIO. They used likelihood analysis to determine if there was an association between the hypothesised iron-loading locus and an increased dietary iron intake that determines TS and unsaturated iron-binding capacity. They studied 236 members of 36 African families. Each selected family contained a proband with iron overload. The model that they presented stated that individuals heterozygous for the hypothesised iron-loading locus would develop iron overload only in conjunction with increased dietary iron but that homozygotes would do so with normal dietary iron. Moyo *et al* (1997b) tested this hypothesis by studying husband and wife pairs from rural Zimbabwe. The spouse pairs lived under the same environmental conditions and would drink similar amounts of beer and therefore if there was no genetic involvement, iron parameters would be similar in the husband and wife. Different iron parameters were noted in the spouse pairs and this led to the conclusion that the iron overload

could not be explained by excess dietary iron alone and that genes may be implicated in the pathogenesis of the disease.

The causative gene of AIO has not yet been identified but the *SLC40A1* gene is a potential candidate because of the similarities in the phenotype of AIO and ferroportin disease (Pietrangelo *et al*, 1999). Gordeuk *et al* (2003) screened the *SLC40A1* gene in Africans and African-Americans with primary iron overload. They identified a polymorphism (Q248H) in the heterozygous state in one African-American subject and three Africans. The polymorphism was also present in the general African-American and African populations. Interestingly, it was absent from all Caucasians with and without primary iron overload who were screened. Standing alone this polymorphism does not seem to be associated with increased SF as there were no significant differences in SF levels in heterozygous family members and controls compared to wild type unaffected individuals. However, among African controls heterozygous for the polymorphism there was a trend towards higher SF levels. It is important to note that the Africans also had excess dietary iron intake in the form of traditional beer and this could suggest that the heterozygous Q248H genotype along with excess dietary iron leads to iron overload. This may also indicate that in the presence of other modifier effects, genetic or environmental, the Q248H polymorphism could lead to significant iron loading. The African-American heterozygous individual had the beta-thalassemia trait and an extremely high SF concentration ($>1300 \mu\text{g/l}$) and macrophage iron deposits. A mild beta-thalassemia trait could be modifying the Q248H phenotype resulting in substantial iron overload.

Further research is necessary to identify the elusive iron-loading gene responsible for AIO.

1.1.6.3 Diagnosing AIO

As the gene associated with AIO has not yet been identified, AIO cannot be confirmed using diagnostic testing. Biochemical tests in African patients must be performed if AIO is suspected. The results obtained from these tests can be confirmed with liver biopsy. AIO differs subtly from Type 1 HH in that iron is deposited in the reticuloendothelial cells first prior to iron being deposited in the hepatocytes.

1.1.7 Treatment of Hereditary Haemochromatosis

The most widely used treatment for iron overload disorders is phlebotomy or venesection. Iron chelation and erythrocytapheresis have also been used (reviewed by Barton *et al*, 1998) but phlebotomy is the most efficient, safest and cheapest treatment. Almost all HH patients benefit from phlebotomy, irrespective of their genotype. In the case of some children and adolescents with severe iron overload, or juvenile haemochromatosis, or men and people with large body mass, a more aggressive phlebotomy regimen is required (1.5 to 2 units per week). Phlebotomy is a safe treatment for older HH patients but some patients are unable to undergo repeated bloodletting and complications can develop. Patients with severe, refractory anaemia should avoid therapeutic phlebotomy and rather undergo iron chelation therapy (Barton *et al*, 1998).

One unit of blood is usually removed from HH patients during each phlebotomy session. This restores normal transferrin and ferritin levels. SF and hepatic iron levels give a good estimate of the amount of phlebotomy required for iron depletion (Witte *et al*, 1996). The agreed upon SF levels for initiation of venesection are 300 µg/l in men and 200 µg/l in women depending on their reproductive status (Witte *et al*, 1996). On average, people who have higher SF have more severe iron overload and need more phlebotomies. In patients with a SF level exceeding 1000 µg/l before treatment, it is sufficient to quantify the SF every four to eight weeks during the initial weeks of treatment. If a patient has received many phlebotomies and in patients with mild iron overload at the onset of treatment, SF levels must be checked more regularly. Once the SF level is 100 µg/l or less, SF levels for each patient must be quantified after each additional one or two treatments. Monitoring haemoglobin and haematocrit, rates of recovery and mean corpuscular volume, assesses the progress of phlebotomy treatment. Venesection can be arrested when SF levels are 10-20 µg/l or when the haemoglobin concentration is 110 g/l or the haematocrit is less than 0.33 for more than three weeks (in patients without chronic anaemia). At these levels mild iron deficiency has been induced and potentially pathogenic excess iron has been removed. After iron depletion, the haemoglobin and haematocrit levels return to within the normal range but the SF levels must be maintained at 50 µg/l or less. The number of annual phlebotomies necessary to maintain SF levels vary in patients but on average, men require removal of 3 to 4 units while women need only 1 or 2 units removed (Barton *et al*, 1996). Some patients do not require any further phlebotomies but SF levels must still be monitored each year.

Patients presenting with manifestations of late disease should undergo the same treatment as individuals not afflicted with haemochromatosis in the general population.

1.1.8 Prevalence of Common *HFE* Mutations C282Y and H63D

Since the discovery of the *HFE* gene, and the two common mutations associated with haemochromatosis (Feder *et al*, 1996), various researchers have attempted to elucidate the frequency of these mutations worldwide. Table 1.1 outlines the allele frequencies reported by a few of these researchers in the general populations from various countries. Genotypes have been reported for various populations and it seems that the C282Y mutation is most prevalent in populations of European descent. In Europe this mutation occurs more frequently in the North than in the South, with the highest frequency being observed in Ireland (Byrnes *et al*, 2001) and this implies a Celtic origin for this mutation. The variant allele is absent from the African, Asian and Australasian populations but is present at very low frequency in the Americas with the exception of the European immigrant population of north America (Merryweather-Clarke *et al*, 1997).

The H63D variant is more common than the C282Y variant. Its presence is more widespread and it occurs more frequently in countries bordering the Mediterranean (Merryweather-Clarke *et al*, 1997; Roth *et al*, 1997). This variant is observed on a shorter haplotype and it is hypothesised that it predates the C282Y variant. The H63D haplotypes observed in the Asian populations differ from those in Europe and this mutation seems to have arisen in Europe and in Asia (Rochette *et al*, 1999).

Table 1.1. Allele frequencies of the H63D and C282Y variants in various populations

Population	Allele Frequency		Reference
	H63D	C282Y	
Europe			
Irish	0.150	0.110	Byrnes <i>et al</i> , 2001
Scottish	0.148	0.097	Campbell <i>et al</i> , 2003
Welsh (South)	0.153	0.082	Jackson <i>et al</i> , 2001
Estonians	0.136	0.035	Parlist <i>et al</i> , 2001
Germans	0.148	0.039	Merryweather-Clarke <i>et al</i> , 1997
Greeks	0.135	0.013	Merryweather-Clarke <i>et al</i> , 1997
Spanish	0.263	0.032	Merryweather-Clarke <i>et al</i> , 1997
Asia			
Chinese (Hong Kong)	0.028	0	Merryweather-Clarke <i>et al</i> , 1997
Taiwanese Aborigines	0	0	Merryweather-Clarke <i>et al</i> , 1997
Indonesians	0.028	0	Merryweather-Clarke <i>et al</i> , 1997
Indian Subcontinent			
North Indians	0.091	0	Garewal <i>et al</i> , 2005
Sri Lankans	0.092	0	Merryweather-Clarke <i>et al</i> , 1997
Africa			
Gambians	0.013	0	Merryweather-Clarke <i>et al</i> , 1997
Senegalese	0	0	Merryweather-Clarke <i>et al</i> , 1997
Kenyans	0.013	0	Merryweather-Clarke <i>et al</i> , 1997
Nigerians	0.019	0	Merryweather-Clarke <i>et al</i> , 1997
Zambians	0.007	0	Merryweather-Clarke <i>et al</i> , 1997
Algerians	0.089	0	Roth <i>et al</i> , 1997
Ethiopians	0.094	0	Roth <i>et al</i> , 1997
Middle East			
Saudi Arabians	0.085	0	Merryweather-Clarke <i>et al</i> , 1997
Americas			
Mexicans	0.065	0	Merryweather-Clarke <i>et al</i> , 1997
Jamaicans	0.022	0.011	Merryweather-Clarke <i>et al</i> , 1997
Vancouver Island Indians	0.014	0.014	Merryweather-Clarke <i>et al</i> , 1997
Australasia			
Papua New Guineans	0	0	Merryweather-Clarke <i>et al</i> , 1997
Australian Aborigines	0	0	Merryweather-Clarke <i>et al</i> , 1997
Vanatuans	0.006	0	Merryweather-Clarke <i>et al</i> , 1997

Abbreviations: H, histidine; D, Aspartic acid; C, cysteine, Y, tyrosine. Adapted from Merryweather-Clarke *et al*, 1997.

1.1.9 Haemochromatosis in the Asian Indian Population

Very few cases of HH have been reported in the Asian Indian population and the literature on this disease in India is very sparse. Garewal *et al* (2005) reported the allele frequency of the C282Y and H63D *HFE* mutations in 60 control subjects and 215 patients with beta thalassemia trait from North India. No individuals presented with the C282Y variant. The H63D variant was observed in the homozygous state in three individuals. There was not a statistically significant difference in iron parameters between the H63D homozygotes and patients with the wild type *HFE* gene. The authors also determined that the H63D haplotype was identical to that of Europeans indicating that this variant originated in Europe and not Asia.

Similar results were reported in another study in the north Indian population. Dhillon *et al* (2007) aimed to identify the frequency of primary iron overload and C282Y, H63D and S65C *HFE* mutations in 100 healthy control individuals and 236 patients with various liver disorders in north India. None of the control subjects were iron loaded and only 17 of the chronic liver disease patients presented with iron overload. Interestingly, iron deficiency was observed in 26% of the control individuals. The authors reported that primary iron overload was rare in India and suggested that the high frequency of iron deficiency anaemia in the Indian population could explain this. The C282Y and S65C mutations were not observed in their study. The H63D mutation was observed with an allele frequency of 13.98% (12% in controls and 14.8% in patients) but none of the H63D homozygous individuals presented with iron overload.

Wallace *et al* (2005) identified a 36-year-old female of Sri Lankan descent with ferroportin disease. The patient was heterozygous for the previously identified *SLC40A1* V162del mutation (Devalia *et al*, 2002; Cazzola *et al*, 2002; Roetto *et al*, 2002b; Wallace *et al*, 2002). This was the first reported case of this disease on the Indian subcontinent and the first time that a mutation in the *SLC40A1* gene had been reported associated with iron overload in India. The authors suggested that because they had identified the mutation in a region where iron overload is very rare, and not well classified, this mutation or others within the *SLC40A1* gene warranted further investigation. They stated that *SLC40A1* mutations could possibly be causing unexplained primary iron overload on the Indian subcontinent.

These results indicate that HH in the Indian population is of the non-*HFE* type but further analysis is necessary to determine the exact defect responsible for HH in Indians. Discovery of the aberrant gene would not only assist in the early diagnosis and treatment of Indian patients but could assist in explaining the high variability of the HH phenotype observed worldwide.

1.2 Iron Homeostasis

1.2.1 Iron Distribution and Circulation

On average, adult males have 35-45 mg/kg of total body iron and premenopausal women have slightly lower stores (approximately 35 mg/kg). The majority of total body iron is incorporated into haem proteins, particularly haemoglobin (60%) and myoglobin (10%-15%). Approximately 10% of iron is found in enzymes and cytochromes but less than 1% is in the plasma bound to transferrin (Andrews, 1999). Transferrin is an 80kD protein with two iron-binding sites (Aisen *et al*, 2001). About 80% of transferrin-bound iron (TBI) is transported to the bone marrow and utilised in the production of haemoglobin in erythroid cells (Conrad *et al*, 1999). When intracellular iron exceeds the cells requirements, iron is stored in ferritin (approximately 30%) (Conrad *et al*, 1999). Ferritin is found within the hepatocytes as well as the reticuloendothelial macrophages and can accommodate 4000-4500 iron atoms (Aisen *et al*, 2001). Only about 4 mg of iron is bound to transferrin and erythrocytes require approximately 20 mg per day. The majority of iron required for haemoglobin synthesis is received from the recycling of senescent red blood cells by reticuloendothelial macrophages (May *et al*, 1995).

1.2.2 Overview of Dietary Iron Uptake

Under proper homeostatic regulation 1 to 2 mg of iron is lost daily through sweating and sloughing off of skin and intestinal cells. This is replenished by dietary iron intake. Because the body has no physiologic pathway that regulates iron excretion, intestinal absorption from the duodenum and jejunum enterocytes plays the major role of regulating body iron stores.

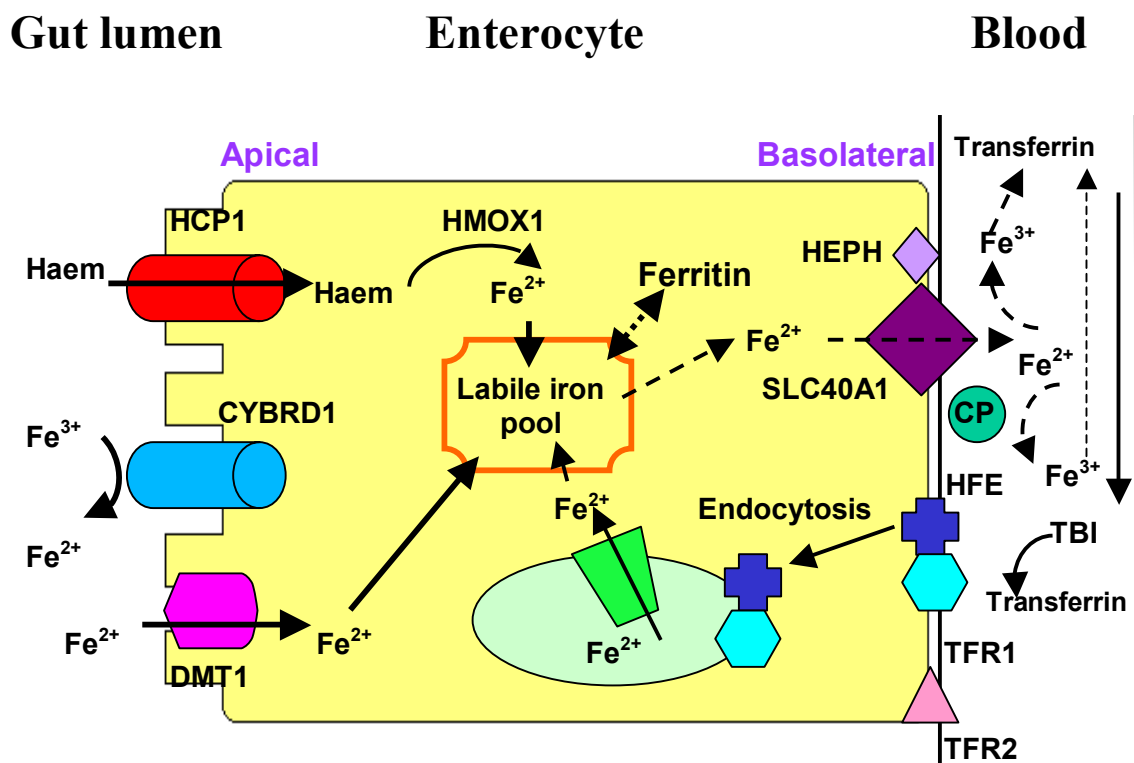
Iron exists in two main forms, the ferric (Fe^{3+}) and the ferrous (Fe^{2+}) form. Before iron can be absorbed, it must be reduced from the ferric to the ferrous form at the apical membrane of the enterocytes. It is believed that the ferrireductase cytochrome b reductase 1 (CYBRD1), also known as duodenal cytochrome b (DCYTB), performs this function (McKie *et al*, 2001). Targeted disruption of the *Cybrd1* gene encoding the mouse homolog of *CYBRD1* does not cause an iron-deficient phenotype (Gunshin *et al*, 2005) suggesting that *Cybrd1* is not essential for iron uptake in the mouse and that possibly other ferrireductases still remain to be identified in humans. Once in the ferrous form iron is transported across the apical membrane into the enterocyte by divalent metal transporter-1 (DMT1), also known as divalent cation transporter-1 (DCT1) or natural resistance-associated macrophage protein 2 (NRAMP2), a proton-coupled divalent cation transporter (Fleming *et al*, 1997; Gunshin *et al*, 1997).

Haem from myoglobin and haemoglobin found in food enters the enterocytes through a different pathway than inorganic iron. Initially, haem needs to be enzymatically cleaved from haemoglobin in the intestinal lumen. The cleaved haem then enters the enterocyte as a metalloporphyrin (reviewed by Anderson *et al*, 2005). It is believed that the recently identified haem carrier protein-1 (HCP1) binds to haem and transports it across the apical membrane of the enterocyte (Shayeghi *et al*, 2005). It is suggested that when HCP1 binds to haem on the cell surface the complex is internalised by receptor-mediated endocytosis and that the resultant endosomal vesicle progresses to the endoplasmic reticulum (ER) (Shayeghi *et al*, 2005). Haem oxygenase-1 (HMOX1) is present on the ER surface and liberates iron from haem. Studies with HCP1 indicate that iron stores post-transcriptionally control haem transport but this exact mechanism has not yet been fully elucidated (Shayeghi *et al*, 2005).

Iron released from haem or imported *via* DMT1 into the enterocyte cytosol enters the labile iron pool. Iron can either be incorporated into ferritin where it is stored within the cell or it can be transported to the basolateral membrane to be exported from the cell. SLC40A1 (ferroportin protein) performs the latter process. Ferroportin works together with membrane-bound hephaestin (HEPH) and serum ceruloplasmin (McKie *et al*, 2000). HEPH is a ferroxidase and homologous to ceruloplasmin, a multi-copper oxidase with ferroxidase activity, which functions in nonintestinal cells. Ceruloplasmin does not transport the iron but it is thought to assist in the release of iron from ferroportin into the blood where it oxidises ferrous iron to ferric iron for binding to transferrin (Harris *et al*, 1998). HEPH is not a transporter either but facilitates the export of iron from the enterocyte. HEPH oxidises ferrous

iron, and releases it into the bloodstream where it binds to transferrin (Harris *et al*, 1998; McKie *et al*, 2000). An overview of dietary uptake is shown in Figure 1.1.

Figure 1.1. Schematic representation of dietary iron uptake



Legend to Figure 1.1.

Dietary iron is reduced by CYBRD1 from the ferric form (Fe³⁺) to the ferrous form (Fe²⁺). Fe²⁺ is transported across the apical membrane by DMT1. In the gut lumen haem is enzymatically cleaved from haemoglobin and transported into the enterocyte *via* HCP1. HMOX1 releases ferrous iron from haem. The intracellular iron is either stored as ferritin or transported out of the cell by SLC40A1, which is located on the basolateral membrane. HEPH (membrane-bound) and CP (in the plasma) assist in the export of iron by oxidising iron from the ferrous (Fe²⁺) to the ferric (Fe³⁺) form, which subsequently binds to transferrin. Abbreviations: CP, ceruloplasmin; CYBRD1, cytochrome b reductase 1; DMT1, divalent metal transporter-1; Fe³⁺, ferric iron; Fe²⁺, ferrous iron; HCP1, haem carrier protein-1; HEPH, hephaestin; HMOX1, haem oxygenase-1; SLC40A1, solute carrier family 40 (iron-regulated transporter) member 1; TBI, transferrin-bound iron; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2. Adapted from Trinder *et al*, 2002a.

1.2.3 Iron Assimilation

1.2.3.1 Hepatocytes

Within the bloodstream iron is transported to various cells bound to transferrin. Transferrin has a high affinity for binding ferric iron and each transferrin protein can accommodate two iron ions. Two transferrin receptors have been isolated, TFR1 and TFR2. TFR1 is expressed in most cells but TFR2 expression occurs mainly in the liver (Kawabata *et al*, 1999). Transferrin binds strongly to the TFRs and is internalised by TFR-mediated endocytosis. The cell acidifies the inside of the endosome and iron is released from transferrin. The apotransferrin-TFR complex is then recycled back to the cell membrane. The neutral pH of the blood promotes release of apotransferrin from the TFR and transferrin can thus continue to bind more iron ions. The HFE- β 2-microglobulin complex has been reported to modulate the functioning of TFR1. TFR2 expression seems to be regulated by TS and is independent of HFE expression. Non-transferrin bound iron (NTBI) may also be taken up in hepatocytes by DMT1 but the iron must be in the ferrous form and a ferrireductase should therefore be present on the cell membrane (Chua *et al*, 2004). The export of iron from the cell occurs in the same manner as on the basolateral membrane of the enterocyte using ferroportin and the ferroxidase ceruloplasmin.

1.2.3.2 Erythroid cells

Erythroid precursors produce haemoglobin and thus need sufficient iron to do so. The majority of iron comes from phagocytosed senescent red blood cells but a small quantity is from the diet. Iron is assimilated in these erythroid precursors in the same manner as explained above for hepatocytes but only TFR1 is present and iron is released from the internalised endosome in a different manner. DMT1 releases iron from the endosome once it is internalised within the cell. Because iron is in the ferric form when bound to transferrin and DMT1 only binds ferrous iron, an endosomal ferrireductase must be present. The six-transmembrane epithelial antigen of the prostate-3 (STEAP3) has been isolated and has been identified as the endosomal ferrireductase (Ohgami *et al*, 2005) that reduces ferric iron to ferrous iron. STEAP3 is highly expressed in erythroid cells and is localised on transferrin-TFR1 endosomes but is not necessary for efficient iron assimilation in other cell types.

1.2.4 Iron Storage

1.2.4.1 Hepatic iron storage

The liver is the main site of iron storage in the human body. Iron is transported to the hepatocytes bound to transferrin and gains entry into these cells *via* TFRs. If intracellular iron levels exceed the cells needs, iron is stored in ferritin and a small amount is stored as haemosiderin.

Ferritin is a heteropolymer of 24 subunits. The subunits are called H for heavy or heart (where they are mostly expressed) and L for light or liver. Both are necessary for the proper functioning of ferritin. Iron is still bioavailable when it is stored in ferritin. The ferritin molecule stores iron in the ferric form and the H-chain has ferroxidase activity for oxidising ferrous iron. Ferric iron is subsequently stored in the ferritin core. The mechanism by which iron is released from ferritin is not well defined but lysosomal or proteosomal degradation of ferritin may be required for the liberation of iron. Ferric iron may also leave through pores in the ferritin molecule (Aisen *et al*, 2001).

Haemosiderin is not as well defined as ferritin. It is a degradation product of ferritin and is water-insoluble. Iron is not released from haemosiderin as readily as it is from ferritin.

1.2.4.2 Reticuloendothelial iron storage

Reticuloendothelial macrophages acquire iron either through phagocytosing senescent erythrocytes (Deiss, 1983) or through the TFR-transferrin pathway (Testa *et al*, 1991). Iron is released from erythrocyte haem by HMOX1 in the macrophages. The iron is then either stored in ferritin or released into the blood where it is oxidised to the ferric state and transported to other cells bound to transferrin.

1.2.5 Regulation of Iron Homeostasis

As mentioned previously the body has no fixed mechanism for excreting excess iron. Thus intricate regulatory mechanisms or pathways must control the amount of iron that is absorbed and stored to prevent iron overload or anaemia. Within individual cells, iron levels are

controlled through post-transcriptional regulation of the proteins involved in iron uptake and export. Mechanisms that regulate the iron homeostasis of the entire organism depend on iron stores, erythropoietic demand (Roy and Enns, 2000) and hypoxia (Ganz and Nemeth, 2006). The crypt programming model and the hepcidin model have been shown to explain iron regulation.

1.2.5.1 Post-transcriptional control

Iron homeostasis is post-transcriptionally controlled by iron-regulatory proteins (IRP1 and IRP2) (Hentze and Kuhn, 1996). IRPs are RNA binding proteins present in the cytosol of cells. The iron levels within cells regulate various proteins involved in iron homeostasis. IRP1 and IRP2 bind to the iron-responsive element (IRE) in the 5' or 3' untranslated region (UTR) of mRNAs encoding these proteins. IRPs and IREs work in conjunction to sense and respond to changing iron levels within the cell. Depending where the IRE is situated, IRP binding will have a different effect on protein synthesis. For example in iron-deficient cells, binding of IRPs to the ferritin 5'-IRE causes a decrease in ferritin transcription and binding to the 3'-IRE in TFR1 results in a more stable mRNA and thus increased expression. Therefore, more iron is supplied to the cell and the iron-deficiency is corrected (Ganz and Nemeth, 2006). When iron concentrations rise, IRP does not bind and ferritin expression is increased and TFR1 expression decreases and iron uptake decreases. Not all proteins involved in iron homeostasis have IREs but they have been found in ferritin (in the 5'-UTR), TFR1 (3'-UTR), ferroportin (5'-UTR) and DMT1 (3'-UTR).

1.2.5.2 Crypt programming model

Within the crypts of the duodenum are precursor cells that migrate onto the villi and differentiate into enterocytes. These precursor cells are responsible for sensing the body's iron requirements and altering iron absorption accordingly. They are not able to absorb iron from the intestinal lumen themselves but as they migrate up the villi they mature into absorptive enterocytes. The crypt programming model suggests that these precursor cells absorb iron from the plasma and that therefore their intracellular iron levels correspond with the body's iron stores. Thus they are able to regulate the amount of iron absorbed from the lumen as they move up the villi and become absorptive enterocytes at the brush border (Oates *et al*, 2000).

Both TFR1 and TFR2 are present on the basolateral membrane of the precursor cells. They allow for the uptake of transferrin-bound iron (TBI) from the plasma. HFE binds to TFR1 but not to TFR2. Waheed *et al* (1999) postulated that by binding to TFR1 and modulating its expression, HFE could modify the precursor cell's iron sensory function. Experiments have shown that wild type HFE binds to TFR1 competing for TBI binding and lowering TBI uptake. This results in lowered intracellular iron concentrations and ferritin and an increase in the number of transferrin receptors. HFE is thus a negative regulator of TFR1 (Roy *et al*, 1999). The mechanism by which HFE works has not yet been fully elucidated. The results of these experiments are questionable because only HFE was transfected and HFE usually functions *in vivo* bound to β 2-microglobulin. In fact it has been reported that when HFE and β 2-microglobulin are overexpressed in cells, TFR1 recycling is enhanced and more receptors are expressed at the cell membrane, which produces an increase in iron uptake (Waheed *et al*, 2002). In *HFE*-related haemochromatosis it has been observed that duodenal crypt cells and macrophages are spared from iron loading and in fact are iron poor (Montosi *et al*, 2000; Philpott, 2002). In the *HFE*-knockout mouse TBI uptake into the enterocytes is also impaired (Trinder *et al*, 2002b). These results taken together could indicate that normal HFE function is necessary to enhance TBI uptake from the plasma by inducing TFR1 expression or by inhibiting iron export from the cell *via* ferroportin. Aberrant HFE functioning in the precursor (crypt cells) therefore causes them to lose their sensory function and results in aberrant iron regulation.

1.2.5.3 Hepcidin model

Hepcidin is a peptide hormone that is produced in the liver (expressed mainly in hepatocytes) and is excreted in the urine (Krause *et al*, 2000; Park *et al*, 2001). It exhibits antimicrobial properties and is thought to be an important regulator of iron homeostasis.

In an experiment performed by Nicolas *et al* (2001) the authors attempted to create *USF2* knockout mice. These mice developed severe iron overload similar to that observed in HH patients. Further analysis revealed that a recombination event had in fact removed both the *USF2* and *HAMP* genes and that hepcidin deficiency was responsible for the iron overload observed. In humans, the most severe form of haemochromatosis results from disruption of the *HAMP* gene encoding hepcidin (Roetto *et al*, 2003). Conversely, overexpression of hepcidin-1 results in severe iron-deficiency anaemia in transgenic mice (Nicolas *et al*, 2002a).

Hepcidin thus negatively regulates iron absorption, recycling and release from stores. Hepcidin expression is decreased in response to hypoxia and anaemia irrespective of the iron load. In humans and mice with inflammation, hepcidin levels increase implicating it in the causation of anaemia of chronic disease (Nicolas *et al*, 2002b).

It has been noted that hepcidin regulates iron efflux from cells by binding to ferroportin and internalising it (Nemeth *et al*, 2004a). It is hypothesised that when hepcidin levels rise in response to iron overload or inflammation, iron export from macrophages and intestinal enterocytes is decreased. And that under iron deficient conditions or HH when hepcidin expression is decreased, iron is released from the intestinal cells and macrophages because ferroportin is able to function normally (Siah *et al*, 2006).

Researchers have noted that in patients with haemochromatosis due to mutations in *HFE*, *TFR2* and *HJV*, urinary hepcidin is decreased despite the presence of excess iron. This could indicate that hepcidin expression is modulated in some way by these proteins (reviewed by Ganz and Nemeth, 2006). How HFE and TFR2 may regulate hepcidin is unknown and needs to be further investigated. Babitt *et al* (2006) have shown how HJV regulates hepcidin expression and how mutations in this gene affect this regulation. They have shown that BMP up-regulates hepcidin expression in hepatocytes and that when HJV is mutated or not present (HJV knockout mice) this up-regulation is not as effective. They have reported that HJV is a coreceptor of the BMP signalling pathway and positively regulates hepcidin expression. Although this helps to explain how *HJV* and *HAMP* mutations cause the same disease (juvenile haemochromatosis), how hepcidin regulates iron absorption from the intestine in response to body iron stores needs to be explored further.

1.3 Genes Involved in Iron Homeostasis

Numerous genes are involved in maintaining the iron levels in the body. These genes perform various functions in iron metabolism and homeostasis. Several genes have been mentioned in the previous sections but only the genes screened for our study will be discussed further. These include the haem oxygenase-1 (*HMOX1*), high-iron (*HFE*), hepcidin anti-microbial peptide (*HAMP*), solute-carrier family 40 (iron-regulated transporter) member 1 (*SLC40A1*),

cytochrome b reductase 1 (*CYBRD1*) and hemojuvelin (*HJV*) genes, listed in chronological order according to when they were isolated.

1.3.1 Haem Oxygenase-1 (*HMOX1*) Gene

HMOX1 (OMIMTM +141250) is a protein that has an important function in haem catabolism. It cleaves the porphyrin ring of haem into carbon monoxide (CO), ferrous iron and biliverdin (Tenhunen *et al*, 1969). Biliverdin is subsequently converted into bilirubin by biliverdin reductase. Yoshida *et al*, (1988) isolated the *HMOX1* gene. Because HMOX1 activity is increased by haem, they increased haem oxygenase activity and mRNA expression in human macrophages through hemin treatment. They subsequently produced a cDNA library and, using rat *Hmox1* cDNA, isolated the human *HMOX1* cDNA. *HMOX1* contains five exons and encodes a peptide that contains 288 amino acids and has a molecular mass of more than 32 kD. The activity of HMOX1 in the liver and other organs is notably increased in the presence of hemin or haemoglobin. This induction has been shown to be due to binding of haem to a translational repressor Bach1 that results in increased expression of *HMOX1* (Ogawa *et al*, 2001).

Kutty *et al* (1994) localised *HMOX1* to chromosome 22q12 using the fluorescence *in situ* hybridisation (FISH) technique. HMOX presents as two isozymes i.e. HMOX1 and HMOX2. HMOX1 is an inducible protein whereas HMOX2 is expressed constitutively. HMOX1 expression has been noted in the spleen, liver, kidney, and bone marrow and is localised on chromosome 16p13.3. HMOX2 is expressed in the brain, testis, and vascular systems and shares 43% homology with HMOX1. Seroussi *et al* (1999) mapped the mouse *Hmox1* gene to chromosome 8 using FISH analysis.

HMOX1 has a hydrophobic sequence at the C-terminal end, which is involved in binding to the microsomal membrane. When HMOX1 is treated with trypsin, the C-terminal hydrophobic region is removed but the protein retains its function and becomes water soluble (Yoshida *et al*, 1991). The rat and human HMOX1 proteins share approximately 80% homology (Yoshida *et al*, 1988). The inner portion in the F helix of the rat *Hmox1* (Pro-126-Lys-149) is called the haem oxygenase signature and highly conserved in HMOX1 isolated from most species. It is thought that this portion of the protein plays an important role in HMOX1 activity. Amino acids with a separable side chain are common in haem enzymes but

absent from mammalian HMOX1 (Schuller *et al*, 1999). No cysteine residues are present in the HMOX1 peptide sequence but there are six histidine residues, of which five are conserved in rat Hmox1 (Yoshida *et al*, 1988).

Poss and Tonegawa (1997) studied the extent to which HMOX1 activity contributed to iron homeostasis. They produced a Hmox1 deficient mouse model. These mice developed anaemia associated with low serum iron levels but hepatic iron levels were high and caused macromolecular oxidative damage, tissue injury and chronic inflammation. Their results indicate that HMOX1 plays an important role in the expulsion of iron from tissue iron stores.

HMOX1 expression is proposed to act in a cytoprotective manner in many cell types. This seems to be due to the increased production of biliverdin and bilirubin, which are strong antioxidants. A case of oxidative stress causing severe injury in endothelial cells in a patient with HMOX1 deficiency has been reported (Yachie *et al*, 1999).

Wagener *et al* (2003) investigated the role of haem and haem oxygenase in the inflammatory response during wound healing in Wistar rats. Haem accumulated at the edges of the wounds and an increase in adhesion molecule expression and the presence of leukocytes was reported. When the inflammatory process was induced, HMOX1 expression increased as well, especially in infiltrating cells. They concluded that haem might be a physiologic trigger that induces the inflammatory response but that HMOX1 antagonises inflammation by modifying the activities of adhesive cells and cellular infiltration.

1.3.2 High-Iron (*HFE*) Gene

The *HFE* gene (OMIMTM +235200) is situated on chromosome 6p21.3 and contains 7 exons. The gene encodes a 343 amino acid protein (HFE) that resembles the MHC class 1 proteins in sequence and structure. HFE is comprised of 3 extracellular domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$ helices), a transmembrane region and a short intracellular region (Feder *et al*, 1996; Lebrón *et al*, 1998). MHC class 1 proteins have a groove present between the $\alpha 1$ and $\alpha 2$ helices that allow binding of peptides. The groove produced by the HFE $\alpha 1$ and $\alpha 2$ helices is shallower than that in the MHC class 1 proteins and therefore HFE is unable to bind peptides (Lebrón *et al*, 1998). HFE has been detected in different tissues including the liver (sinusoidal lining cells, bile duct epithelial cells and Kupffer cells), duodenum, heart, pancreas, placenta, kidneys,

ovaries, colon, leukocytes, tissue macrophages, circulating monocytes, brain (capillary endothelium) and lungs (Feder *et al*, 1996; Parkkila *et al*, 1997; Parkkila *et al*, 2000).

A study on β 2-microglobulin-deficient mice provided the first indication that HFE could be involved in iron metabolism (De Sousa *et al*, 1994). These transgenic mice developed iron overload in a manner similar to that observed in humans with HH. It had previously been noted that β 2-microglobulin became physically associated with MHC class 1 proteins and therefore indicated that β 2-microglobulin or a β 2-microglobulin-associated protein may be involved in iron homeostasis and/or the pathogenesis of HH.

Simon *et al* (1976) found an association with HH and the HLA-A3 locus but it was not until 1996 when the gene was localised to 6p21.3 and isolated (Feder *et al*, 1996). The gene was named *HFE* and it was found in later studies that it indeed did associate with β 2-microglobulin (Feder *et al*, 1997).

The HFE- β 2-microglobulin complex binds to TFR1 and modulates its expression and presentation at the cell membrane. In this way, HFE may regulate the amount of TBI brought into the cell. The HFE- β 2-microglobulin complex actually competes with TFR1 for binding to TBI and can reduce the binding affinity of TFR1 10-fold (Parkkila *et al*, 1997, Feder *et al*, 1998). In the duodenum, HFE is confined to the crypt cells and could possibly be involved in the regulation of iron absorption. The C282Y variant disrupts the interaction between HFE and β 2-microglobulin and less HFE is presented at the cell membrane. The loss of HFE regulation on TFR1 results in the increased iron absorption characteristic of type 1 HH.

Animal studies have been performed to confirm the involvement of HFE in iron homeostasis. The *Hfe*-knockout mouse exhibited an iron overload phenotype very similar to that in HH patients (Zhou *et al*, 1998) and this led researchers to conclude that HFE is involved in iron homeostasis and the pathogenesis of haemochromatosis (also see Section 1.1.1).

1.3.3 Hepcidin Antimicrobial Peptide (*HAMP*) Gene

Two separate research groups isolated the *HAMP* gene (OMIMTM *606464). Krause *et al* (2000) isolated a cDNA encoding hepcidin by biochemical purification of blood ultrafiltrate using a cysteine alkylation assay and mass spectrometry followed by sequence and reverse

transcriptase PCR (RT-PCR) analysis and rapid amplification of cDNA ends (RACE). The authors named the peptide liver expressed antimicrobial peptide 1 (LEAP1). Park *et al* (2001) isolated an antimicrobial peptide from human urine and named it hepcidin because it originates in the liver and exhibits antimicrobial properties.

HAMP is comprised of three exons and is localised on chromosome 19q13. The active protein is encoded entirely by exon 3 (Park *et al*, 2001; Krause *et al*, 2000). The *HAMP* gene encodes a propeptide of 84 amino acids that after enzymatic cleavage produces mature peptides of 20, 22 and 25 amino acids (Park *et al*, 2001). The active peptides are rich in cysteines, which form intramolecular bonds that stabilise the β -sheet structure. Hepcidin expression has been detected at very high levels in the liver. Moderate levels have been noted in the heart and brain but very little expression is reported in the lung and other tissues (Krause *et al*, 2000)

Various animal models have been developed in order to establish the function of hepcidin and how it is regulated. Pigeon *et al* (2001) isolated the cDNA encoding mouse *Hamp* in the liver. The protein shares 54% homology with human hepcidin and expression is increased in response to iron overload and lipopolysaccharide stimulation. Iron levels and inflammation regulate hepcidin expression. Nemeth *et al* (2004b) noted that in the mouse hepcidin is regulated by interleukin-6 (IL6) under inflammatory conditions but that IL6 is not necessary for hepcidin regulation by iron. Nicolas *et al* (2001) noted the importance of hepcidin in iron regulation through studying mice in which the *Usf2* gene was disrupted. The *Usf2* gene lies upstream from and is very close to the *Hamp* gene. No hepcidin expression was detected in these mice and they developed an iron overload phenotype as seen in HH patients and *Hfe*-knockout mice. In a later study, Nicolas *et al* (2002a) produced a murine model in which hepcidin was overexpressed and these animals subsequently developed severe microcytic hypochromic anaemia. These findings lead the researchers to conclude that hepcidin is a key regulator of iron absorption. Disruption of the *HAMP* gene leads to juvenile haemochromatosis, which is discussed in Section 1.1.2.

Hepcidin is believed to act as a negative regulator of iron release from macrophages and enterocytes in the duodenum. Hepcidin expression is increased under conditions of iron overload but this does not occur in patients homozygous for the C282Y variant in the *HFE* gene (Bridle *et al*, 2003) or in *Hfe*-knockout mice (Ahmad *et al*, 2002).

The urinary hepcidin levels of patients with *HFE*-associated haemochromatosis as well as those with *HJV*- and *TFR2*-associated haemochromatosis are very low. In fact the urinary hepcidin levels in patients with *HJV* associated Type 2 haemochromatosis are virtually undetectable. *Hfe*- (Muckenthaler *et al*, 2003; Nicolas *et al*, 2003), *Tfr2*- (Kawabata *et al*, 2005) and *Hjv*-deficient mice (Niederkofler *et al*, 2005; Huang *et al* 2005) do not induce hepcidin expression either. This indicates that these genes all function on the same hepcidin regulatory pathway and that *HJV* is the principal hepcidin regulator.

1.3.4 Solute-carrier Family 40 (iron-regulated transporter) Member 1 (*SLC40A1*) Gene

SLC40A1 was formerly known as solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 (*SLC11A3*) gene and is also called ferroportin 1 (FPN1), iron-regulated transporter 1 (IREG1) and metal transporter protein-1 (MTP1) (OMIMTM *604653).

Reports of the isolation of an iron exporter were described by three separate research groups in the same year. Positional cloning was used by Donovan *et al* (2000) to identify the gene responsible for hypochromic anaemia in the mutant zebrafish 'weissherbst'. The gene was named *fpn1* and *fpn1* cDNAs were also isolated from mouse liver and human placenta by RT-PCR. McKie *et al* (2000) used a subtractive cloning technique and PCR analysis to isolate FPN1 from human and mouse duodena. They decided to name the protein IREG1. An iron-responsive protein affinity column was utilised by Abboud and Haile (2000) to isolate mRNAs that contained functional IREs. The protein that they isolated was subsequently named MTP1.

The *SLC40A1* gene is located on chromosome 2q32 and consists of eight exons that encode a 571 amino acid peptide. Expression has been noted in the placenta, liver, spleen, and kidneys of humans (Donovan *et al*, 2000). Initially McKie *et al* (2000) reported 10 transmembrane domains in the *SLC40A1* protein but Liu *et al* (2005) have noted that there are in fact 12 in this protein. Iron absorption in the duodenum is initiated by the uptake of ferrous iron by DMT1 in the duodenal enterocytes. The iron is transported across the cell and is transferred out of the cell across the basolateral membrane by *SLC40A1*. *SLC40A1* is localised on the basolateral membrane of all polarised cells, including duodenal enterocytes, hepatocytes, placental trophoblasts and cells of the central nervous system (CNS), and is an essential iron exporter. *SLC40A1* is the only known mechanism of iron export and deletion of *SLC40A1* is

lethal in transgenic mice whereas inactivation of *SLC40A1* by a conditional knockout results in excess iron being stored in macrophages, hepatocytes and enterocytes (Donovan *et al*, 2005). Once iron is transported out of the cell in the ferrous form, it must be reduced to the ferric form for incorporation into transferrin. Ceruloplasmin fulfils this function in nonintestinal cells and its homolog HEPH is the ferroxidase in the intestine.

Expression of *SLC40A1* is controlled in two ways: iron levels and hepcidin. In the 5' UTR of *SLC40A1* mRNAs there is a functional IRE (McKie *et al*, 2000). When iron levels within the cell decrease, an IRP binds to the IRE and decreases mRNA stability and expression. When iron levels rise, the IRP does not bind and mRNA expression increases.

SLC40A1 mRNA levels are inversely correlated with hepcidin mRNAs. When iron levels are high, hepcidin regulates ferroportin functioning by binding to it on the basolateral membrane and internalising it. Hepcidin then induces the destruction of *SLC40A1* in the internalised vesicles thus decreasing the amount of *SLC40A1* on the cell membrane (Nemeth *et al*, 2004a). When iron levels return to normal, hepcidin does not bind and *SLC40A1* functions normally.

Variants that inactivate *SLC40A1* function have been associated with an autosomal dominant form of haemochromatosis also known as the ferroportin disease. The phenotype of this disorder is highly variable and the heterogeneity and range of *SLC40A1* mutations seem to be the cause of this variation (see Section 1.1.4).

1.3.5 Cytochrome b Reductase 1 (*CYBRD1*) Gene

In 2001 a candidate mammalian ferric reductase was isolated from hypotransferrinaemic mice by McKie *et al* (2001) using a subtractive cloning procedure. They named the gene duodenal cytochrome b (*DCYTB*) but it is also called *CYBRD1* (OMIMTM *605745). *CYBRD1* was mapped to chromosome 2q31 by the International Radiation Hybrid Mapping Consortium and consists of four exons. The gene encodes a 4 254 bp mRNA molecule that undergoes splicing to produce 3 alternative transcripts. The protein consists of 286 amino acids, includes six transmembrane domains and four conserved histidine residues and is highly hydrophobic (McKie *et al*, 2001). *CYBRD1* is highly expressed in the brush border of enterocytes near the tip of the villus in the duodenum and its expression is highly dependent on iron levels

although its mRNA does not possess an IRE in either its 5'- or 3'-UTR (McKie *et al*, 2001). CYBRD1 is expressed at very low levels in the liver and spleen.

CYBRD1 shares 40-50% homology with cytochrome b561, an enzyme involved in the regeneration of ascorbic acid from dehydroascorbate. The predicted binding sites for ascorbic acid and dehydroascorbic acid are highly conserved within CYBRD1 (Latunde-Dada *et al*, 2002).

In the intestinal lumen iron is found in the ferric form. The main iron importer on the duodenal enterocytes, DMT1, is only able to transport ferrous iron across the cell membrane into the cells. CYBRD1 possesses ferrireductase activity and is present on the cell membrane and reduces ferric iron to ferrous iron prior to transport into the enterocyte.

As mentioned previously CYBRD1 is regulated by iron. CYBRD1 mRNA and protein levels were increased in the duodena of mice when iron levels were low (McKie *et al*, 2001). CYBRD1 expression is up-regulated under hypoxic conditions in the liver but hypoxia does not seem to affect CYBRD1 in the liver or spleen (Latunde-Dada *et al*, 2002). Zoller *et al* (2003) noted a decrease in CYBRD1 expression, and thus activity, in the human duodena from patients with iron deficiency anaemia. They also found that CYBRD1 activity was up-regulated posttranslationally in haemochromatosis patients with *HFE* variants. Muckenthaler *et al* (2003) also found altered CYBRD1, SLC40A1 and hepcidin expression in an *Hfe*-deficient mouse model. They proposed that increased duodenal iron absorption, characteristic of haemochromatosis, could be due to the inappropriate regulatory cues from the liver, possibly involving CYBRD1.

1.3.6 Hemojuvelin (*HJV*) Gene

The most common form of JH was linked to chromosome 1q. Because no gene regulating iron homeostasis was known to exist on chromosome 1q, positional cloning strategies were used to identify this putative JH gene. Papanikolaou *et al* (2004) cloned the putative JH gene and named it hemojuvelin (*HJV*) (OMIMTM *608374).

The *HJV* gene is located on chromosome 1q21 and its four exons span 4 265 nucleotides. The primary 2.2 kb transcript has five spliced isoforms. The longest transcript encodes a 426

amino acid peptide. The protein possesses multiple protein motifs and shows homology to the repulsive guidance molecule (RGM) that suggests that HJV may function as a membrane-bound receptor or as a secreted polypeptide hormone. HJV expression is detected in the liver, brain, heart, kidney, pancreas, skeletal muscle, oesophagus and parts of the colon (Papanikolaou *et al*, 2004; Rodriguez-Martinez *et al*, 2004).

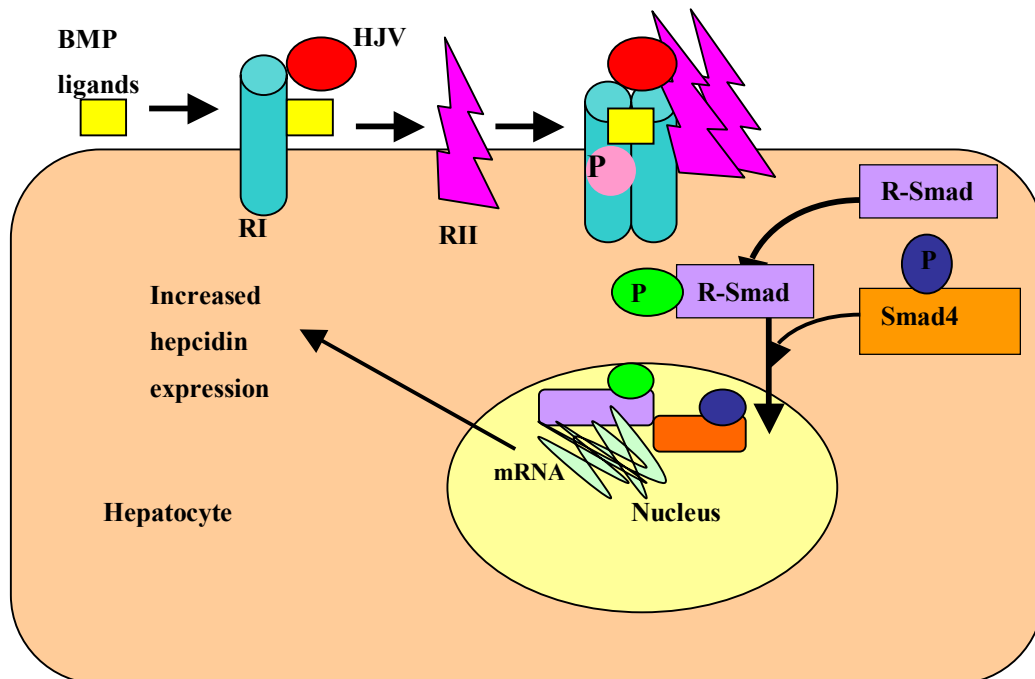
Numerous animal models have been studied to elucidate HJV function and regulation. Niederkofler *et al* (2005) noted *Hjv* expression in the mouse liver. Iron overload was observed in *Hjv*-knockout mice and no hepcidin expression was observed in response to dietary or injected iron. Up-regulation of hepcidin expression still occurred in the *Hjv*-knockout mice in response to acute inflammation, which was induced by lipopolysaccharide or IL6 and Tnf- α . In wild type mice, *Hjv* was down regulated in the liver in response to inflammation but not in skeletal muscle. The researchers concluded that *Hjv* is an important sensor of iron levels and that its inactivation leads to iron overload and also that down-regulation of *Hjv* during the inflammatory response could temporarily eliminate *Hjv*'s ability to sense iron levels (Niederkofler *et al*, 2005).

Huang *et al* (2005) also produced a *Hjv*-knockout mouse model and found that iron rapidly accumulated in the liver, pancreas and heart but that there were decreased iron levels in the spleen. Hepcidin expression in the liver was decreased and ferroportin expression was increased in the intestinal epithelial cells and macrophages. The authors concluded that JH could be the result of decreased hepcidin regulation and increased ferroportin expression. The increase in ferroportin expression would lead to more iron liberation from macrophage and intestinal epithelial cells and higher TS in the bloodstream.

HJV is proposed as the key modulator of hepcidin expression. This is due to the fact that HJV is expressed in the same tissue as hepcidin (liver) and because the urinary hepcidin level in JH patients with *HJV* variants is extremely low (Papanikolaou *et al*, 2004). HJV acts as a coreceptor of the BMP signalling pathway and assists in the positive regulation of hepcidin. Babitt *et al* (2006) proposed that HJV binds to BMP Type I and II receptors, forming an active complex. The Type II receptors subsequently phosphorylate the Type I receptors, which then phosphorylate three receptor regulated Smads namely, Smad1, 5 and 8. A common Smad, Smad 4, forms a complex with the phosphorylated Smad peptides and this

entire complex migrates to the nucleus where it increases transcription of hepcidin. A schematic diagram of this regulatory pathway is shown in Figure 1.2.

Figure 1.2. Schematic diagram of the role HJV plays in the regulation of hepcidin expression via the BMP signalling pathway



Legend to Figure 1.2.

HJV forms a complex with BMP ligands, BMP Type I receptors and BMP Type II receptors within the hepatocyte. Within the activated complex Type II receptors phosphorylate Type I receptors, which then phosphorylate receptor-activated Smads. The phosphorylated R-Smads form a complex with Smad4. The Smad complex enters the nucleus where it increases hepcidin expression. Abbreviations: BMP, bone morphogenetic protein; HJV, hemojuvelin; mRNA, messenger RNA; P, phosphorous; RI, BMP Type I receptor; RII, BMP Type II receptor; R-Smad, receptor mediated Smads. Adapted from Babitt *et al*, 2006.

1.4 Objectives of This Study

HH is a very common disorder that is often misdiagnosed because of the vague symptoms presented by patients when the disease first becomes evident. Early diagnosis of this easily treatable condition will assist in preventing the development of the more severe symptoms that manifest as the disease progresses untreated. Identification of genetic variation associated with HH or modifying the disorder will improve diagnosis and ultimately improve the longevity of HH patients.

The aim of this study was to perform mutation analysis of six iron regulatory genes, including the haem oxygenase 1 (*HMOX1*) gene, high-iron (*HFE*) gene, hepcidin antimicrobial peptide (*HAMP*) gene, solute carrier family 40 (iron-regulated transporter) (*SLC40A1*) gene, cytochrome b reductase 1 (*CYBRDI*) gene and hemojuvelin (*HJV*) gene, by performing the following:

- PCR amplification of the promoter and coding regions of the respective genes
- Analysis of the amplified fragments employing heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis, restriction fragment length polymorphism (RFLP) analysis and bi-directional semi-automated DNA sequencing analysis to identify any known and/or novel variation within these genes

The variants observed were analysed to identify possible associations with iron overload and to identify possible modifiers of the HH phenotype observed in a South African family of Indian extraction.

CHAPTER TWO

DETAILED EXPERIMENTAL PROCEDURES

2. Detailed Experimental Procedures

The project has gained ethical approval from the Research and Ethics/Biosafety Committee, Faculty of Health Sciences, Stellenbosch University (Ethics number N04/08/123).

2.1 Subjects

Whole blood samples were collected from 25 members (two probands and 23 unaffected individuals) of a South African family of Indian extraction diagnosed with Classic (Type I) HH. The collaborating clinician was responsible for clinical examination of the probands. The two probands, a brother and sister, were diagnosed with HH and were used for initial screening of the genes under investigation. Secondary factors such as excessive alcohol consumption, hepatitis C infection and beta-thalassemia, that could be causing iron overload, were eliminated. Written informed consent was obtained from all individuals participating in the project.

The family originates from the Kond village approximately 120 km from Mumbai in the Maharashtra State. The village is located in the Raigad (also known as Raigarh) district in the South Western part of India. This family is highly consanguineous and the two probands are the product of a consanguineous relationship between two first cousins.

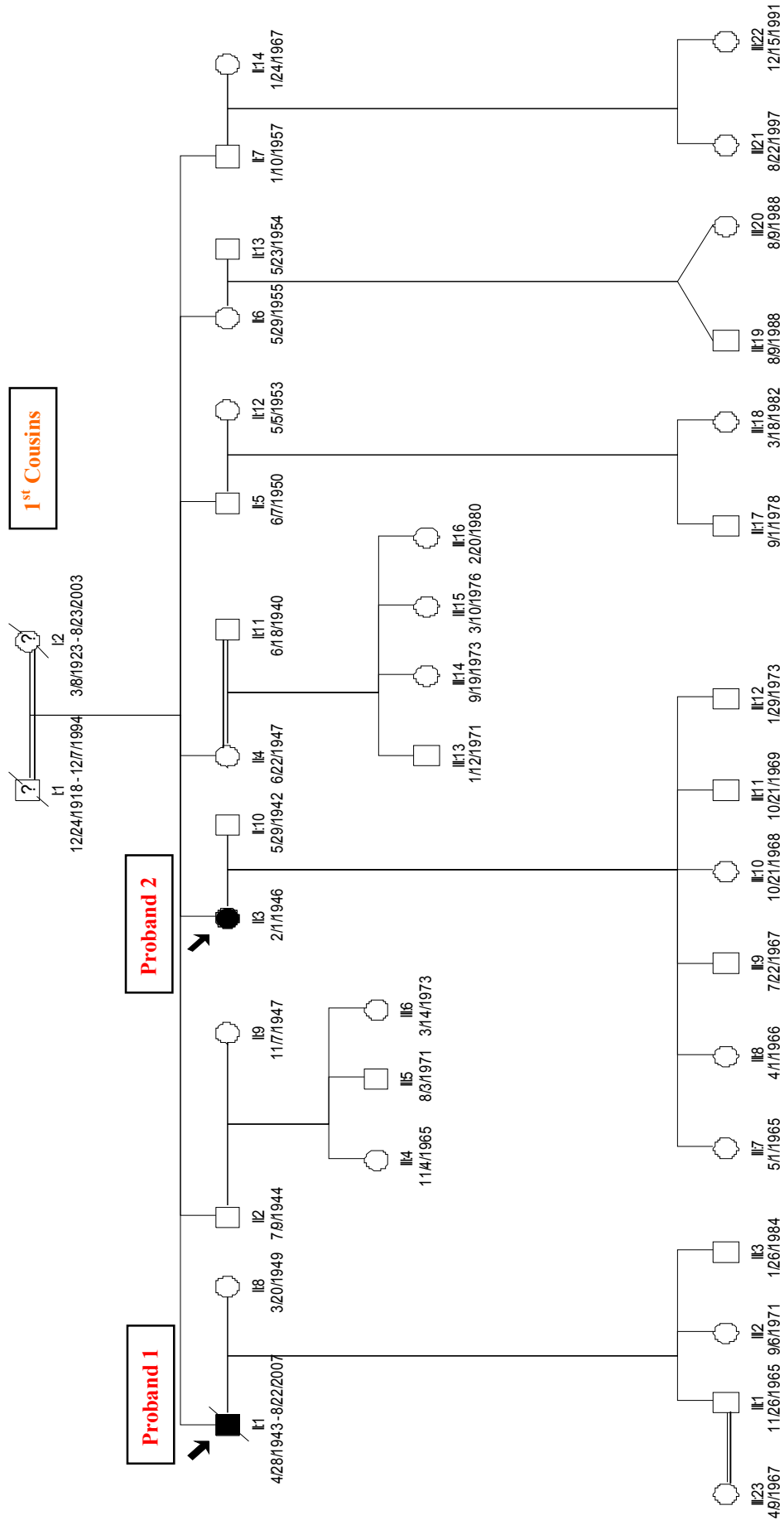
Proband 1 was a 64-year-old male. He presented to the Groote Schuur Hospital at the age of 49 with a history of arthritis involving the hips, knees and hands and vague abdominal symptoms. The patient had also noticed increasing pigmentation of the skin. Upon examination, generalised hyperpigmentation was observed. The patient was a non-insulin-dependent diabetic and there was evidence of arthritis particularly of the proximal interphalangeal joints of the second and third fingers of both hands as well as pain on movement of the knees and hips. Abdominal examination revealed that the patient had slight hepatomegaly and moderate splenomegaly. The patient was subsequently referred for biochemical testing to determine his iron parameters. Biochemical examination revealed an SF level of 5220 $\mu\text{g/l}$ (reference range: 30-300 $\mu\text{g/l}$), TS% of 100% (reference range: 20-50%) and a serum iron of 50.4 $\mu\text{mol/l}$ (reference range: 10-30 $\mu\text{mol/l}$). The patient's alanine aminotransferase (ALT) level was slightly raised at 50.4 $\mu\text{g/l}$ (reference range: 1-40 $\mu\text{g/l}$).

indicating liver injury. X-ray of the fingers revealed juxta-articular osteoporosis as well as joint space narrowing and erosions. A liver biopsy was performed and showed micronodular cirrhosis with massive parenchymal iron deposition. The patient was diagnosed with HH with established cirrhosis, arthritis and diabetes. After the diagnosis the patient commenced fortnightly venesection until he was discharged in the year 2000 with a normal SF level. The management of his arthritis was complicated by the development of a non-steroidal-induced duodenal ulcer. In 2006 he required a total hip replacement. He remained well until approximately 2005 when he developed complications of end-stage cirrhosis including oesophageal varices, ascites, encephalopathy, renal failure and hepatocellular carcinoma.

Proband 2, the sister of proband 1, was a 61-year-old female who presented with hyperpigmentation on her face at the age of 49. Examination in 1999 confirmed mild hyperpigmentation. The patient was not diabetic and did not present any other complications of haemochromatosis. Biochemical examination revealed an SF level of 595 $\mu\text{g/l}$ (reference range: 12-150 $\mu\text{g/l}$) and TS% of 58% (reference range: 20-50%). Her aspartate aminotransferase (AST) level was slightly raised at 51 ng/ml (reference range: 1-40 ng/ml). A liver biopsy revealed hepatic steatosis with hepatic parenchymal iron deposition confirming the HH diagnosis. The patient commenced monthly venesection until her ferritin levels declined to normal levels the following year. She has remained well subsequently.

It was not known if there was a family history of haemochromatosis in this Indian family. The probands' father and paternal grandfather both had dark patches on their skin but were never diagnosed with HH. The father and grandfather had both passed away prior to the initiation of this study so the presence of HH could not be verified. The group of 23 unaffected family members comprised 12 females (52%) and 11 males (48%). The pedigree of the family, with the individuals' respective phenotypes and age, is shown in Figure 2.1. Individuals II:8, II:9, II:10, II:11, II:12, II:13, II:14, III:6, III:11, III:13, III:15 and III:18 did not provide written informed consent and were therefore excluded from the present study. Participating family members were all described as unaffected, but some individuals were young and could be pre-symptomatic disease carriers. In time they could present with HH symptoms.

Figure 2.1. Phenotypic pedigree of the Indian family analysed in this study



2.2 Methods

2.2.1 Body Iron Status

The transferrin saturation percentage (TS%) and the serum ferritin (SF) levels were measured in all of the extended family members according to standard methodology. The collaborating clinician provided the TS% and SF levels of the two probands at diagnosis. The laboratory at which the blood samples were analysed employed reference ranges of 25-35% for TS% and 22-322 µg/l for SF. In the present study iron overload was defined as TS% > 45% (Looker and Johnson, 1988) and/or a SF level exceeding 200 µg/l in females and 300 µg/l in males. Iron status was classified into four groups: iron deficiency (SF level < 20 µg/l); normal SF levels (20-200 µg/l in females, 20-300 µg/l in males); high SF levels (females: > 200 µg/l, males: > 300 µg/l) with a TS% < 45% and high SF levels with TS% > 45%.

2.2.2 DNA Isolation From Whole Blood Samples

All whole blood samples were collected in tubes containing ethylene diamine tetra-acetic acid (EDTA) (all chemicals/reagents and respective suppliers provided in Appendix 1) as the preservative. DNA was isolated from all samples using an adaptation of the Miller *et al* (1988) salting out procedure. The initial step for extraction required the transfer of 10 ml of each whole blood sample to a separate 50 ml Falcon tube (Merck). A volume of 30 ml cold lysis buffer (155 mM ammonium chloride (NH₄Cl), 10 mM potassium hydrogen carbonate (KHCO₃) and 0.1 mM EDTA (C₁₀H₁₆N₂O₈) – pH 7.4) was then added to the sample and it was subsequently placed on ice for 30 minutes and mixed by inversion at 10-minute intervals, allowing for complete lysis of cells. Following this, the sample was centrifuged at 1500 rpm for 10 minutes (Hermle Z 200 A, Labnet, Avanti™ 30, Beckman, GS-15R, Beckman).

The supernatant was discarded and the pellet was washed with 10 ml cold phosphate buffered saline (PBS) [27 mM potassium chloride (KCl), 137 mM sodium chloride (NaCl), 8 mM disodium hydrogen orthophosphate anhydrous (Na₂HPO₄) and 1.5 mM potassium di-hydrogen orthophosphate (KH₂PO₄) – pH 7.3]. The solution was subsequently centrifuged at 1500 rpm for 10 minutes and the supernatant was removed. The pellet was resuspended in 3 ml cold nucleic lysis buffer (10 mM Tris(hydroxymethyl)aminomethane (Tris-HCl) ((CH₂OH)₃CNH₂-

Cl), 400 mM NaCl and 2 mM EDTA – pH 8.2), 1% (w/v) sodium dodecyl sulphate (SDS) and 1.5 mg/ml proteinase K (Roche Diagnostics), and incubated in a water bath at 55°C overnight.

Following overnight incubation, 1 ml saturated 6 M NaCl was added and the solution was shaken vigorously for 1 minute, followed by centrifugation for 20 minutes at 3500 rpm. The supernatant was transferred to a clean Falcon tube, shaken for 15 seconds and subsequently centrifuged at 2500 rpm for 30 minutes. After centrifugation the supernatant containing the DNA was poured into a new Falcon tube. Two times the volume ice-cold ethanol (EtOH) ($\pm 99.9\%$) (v/v) was added and the solution was left at room temperature for 30 minutes to allow the DNA to precipitate.

The precipitated DNA was placed into a clean 1.5 ml tube (Eppendorf) containing 1 ml 70% (v/v) EtOH for the removal of excess salt. The solution was centrifuged at 14 000 rpm (Centrifuge 7417C, Eppendorf) for 10 minutes (4°C). Excess EtOH was carefully removed and the DNA left to air-dry at room temperature. The DNA pellet was dissolved in 200-800 μ l double distilled water (ddH₂O), depending on the pellet size, and then shaken overnight at room temperature and subsequently stored at 4°C. DNA quantity and quality was determined spectrophotometrically (Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, United States of America (USA)))

2.2.3 Polymerase Chain Reaction (PCR) Amplification

PCR amplification was performed for the promoter and coding region of the genes under investigation, including *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* and *HJV*. Oligonucleotide primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3/www.cgi>, 2002) and the reference sequence for each gene as listed in Tables 2.1 – 2.6. Reference sequences were obtained from either the Ensembl (<http://www.ensembl.org>) or GenAtlas (<http://www.genatlas.org>). The reference promoter and coding regions of all the genes with relative positions of the primers designed are depicted in Appendix 2.

A final reaction volume of 25 μ l contained 50 ng DNA, 1 \times ammonium sulphate buffer ((NH₄)₂SO₄) (Fermentas), 10 pmol of each primer [Inqaba Biotech and Integrated DNA Technologies (IDT)], 0.5 U *Taq* polymerase (Fermentas), magnesium chloride (MgCl₂)

(Fermentas) as specified in Tables 2.1 – 2.6 and, unless otherwise stated in the Tables, 0.1 mM of each 2'-deoxynucleotide (dNTP) (dATP, dGTP, dCTP, dTTP) (Fermentas).

PCR amplification was performed in an Applied Biosystems PCR thermocycler (GeneAmp®PCR system 2700). Five different PCR programmes were utilised to amplify the promoters and coding region of the genes as mentioned above. These programmes have been designated programmes A to E and the programme used to amplify a specific amplicon is indicated in Tables 2.1-2.6. These are discussed further below:

Programme A was initiated by a denaturation step at 95°C for 2 minutes. Followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds (Ann1 as specified for each fragment and listed in Tables 2.1 – 2.6) and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 10 minutes.

Programme B was characterised by an initial 2 minute denaturation step at 95°C. Subsequently, there were 10 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds (Ann 1 as listed for each primer set in Tables 2.1 – 2.6) and extension at 72°C for 30 seconds. This was followed by 30 cycles of denaturation, annealing (Ann 2 as listed for each primer set in Tables 2.1 – 2.6) and extension at the same conditions as above. Final extension occurred at 72°C for 10 minutes.

Programme C included an initial denaturation step (95°C for 5 minutes) followed by 35 cycles each consisting of 1 minute at 95°C (denaturation) and 2 minutes at Ann 1 (as specified in Tables 2.1 – 2.6) (annealing). Final extension was at 72°C for 15 minutes.

Programme D included an initial denaturation at 94°C for 5 minutes preceded 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds (Ann 1 as listed in Tables 2.1 – 2.6) and extension at 72°C for 30 seconds. The cycle was completed with an extension step at 72°C for 10 minutes.

Programme E was initiated by denaturation at 95°C for 5 minutes. This was followed by 35 cycles of denaturation at 95°C for 2 minutes and annealing (Ann 1 as specified for each primer set as listed in Tables 2.1 – 2.6) for 2 minutes. Subsequently, an extension step at 72°C for 10 minutes occurred.

Table 2.1. Oligonucleotide primers designed for PCR amplification of *HMOX1* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	Ann 2 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
<i>HMOX1</i> gene promoter region [Reference sequence: ENSG00000100292 (Ensembl)]										
XP1 ^a	GGCCAGTCTCGAACTCAAAG	62	AATCAATTCTGCATGAGGTGG	60	267	A	55		2	0.1
XP2 ^a	AGGACTGTATGAATCATCTG*	56	CAGTGGTGTACACATAAAG	58	343	A	55		1.5	0.1
XP3 ^a	ATACCAGGTGCTGCCAGAGT	62	AGGCTCTGCTGTCTCCAGCT	64	317	A	56		1	0.1
XP4 ^a	TGTGAGGAGGCAAGCAGTC*	60	TCTGAGAAGCTGCAGGCTC*	60	251	B	60	55	1.5	0.1
XP5 ^a	GTGCATCAAGTCCCAAGGG*	60	AACAGCTGATGCCACATTTTC	60	304	N/O				
XP6 ^a	AGACTTTGTTTCCCAAGGGT	58	GCAGGGTTGGAGAGAAAG	60	332	A	55		2	0.1
<i>HMOX1</i> gene coding regions [Reference sequence: NM_002133 (GenAtlas)]										
X1 ^b	CCGCCGAGCATAAATGTG	60	GCACAGGCAGGATCAGAAC	62	300	E	60		1.5	0.1
X2 ^c	CAGCCAGCTTTGTTCACC	62	AACCACTGGTCTGAGCCTTG	62	236	C	60		1.5	0.1
X3A ^c	TAGTGACGGGACGGACAGA	64	CCTTGCGGTGCAGCTCTTCT	64	187	C	60		1.5	0.1
X3B ^c	TGAGCGCAACAAGGAGGCC	64	GGAAGGTGAAGAAGGCCAGG	64	314	C	60		1.5	0.1
X3C ^c	GCCTGGCCTTCTCACCTTC	64	TGGCAGTGTGGAACCTTGG	64	276	C	60		1.5	0.1
X4 ^c	GGACCTGGTAGCATCTCA*	62	GCGAGAACCCTGTCCCTACAG*	68	314	C	60		1.5	0.1
X5A ^c	CCACCTGTTAATGACCTTGC	60	GAAGATGCCATAGGCTCCTT	69	343	C	60		1.5	0.1
X5B ^c	GGAAGGAGCCTATGGCATCT	62	GCTGAGCCAGGAACAGAGTG	64	303	C	60		1.5	0.1
X5C ^c	CACTCTGTTCTGGCTCAGC	64	CTCCTACCGAGCACGCAAGA	64	254	C	60		1.5	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; dATP, 2'-deoxy-adenosine-5'-triphosphate; dCTP, 2'-deoxy-cytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dTTP, 2'-deoxy-thymidine-5'-triphosphate; *HMOX1*, haem oxygenase-1 gene; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; T_m, melting temperature. T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986). References: ^aThis study; ^bF Booley (unpublished data); ^cH Waso (unpublished data). All primers supplied by Inqaba Biotech except * supplied by IDT (Integrated DNA Technologies).

Table 2.2. Oligonucleotide primers designed for PCR amplification of the *HFE* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
<i>HFE</i> gene promoter region [Reference sequence: ENSG0000010704 (Ensembl)]									
HP1 ^a	GGTTTGAGCAAAATCAGGTG	60	GTGCTGAGTTCACCTTCGCAG	62	341	A	55	1.5	0.1
HP2 ^a	CGGAGCTCTGAACCAGCAAT	62	ATAGGTAAGACCATGACACAG	58	305	A	55	1.5	0.1
HP3 ^a	TGAGGTTTGGCTGTATCTG	60	GTCTGTGGCCAAAATATCCTG	62	292	A	57	1.5	0.1
HP4 ^a	CATGTGCCACCTTAGGGAAAT	60	CTGCTCCTAACCCACAGACAC	64	249	A	55	1.5	0.1
HP5 ^a	TTAGTGACAGCCCTTCCGCT	60	AACACCTTCCACAGCCCTTCA	60	383	A	55	1.5	0.1
HP6 ^a	TCTGTGATCCCCTCCCAACT	62	GGGAAAGTAGCTTCGCAATG	60	387	A	55	1.5	0.1
HP7 ^a	GTGCTGGGGTTAGGAGCAG*	64	CTCAGGAGATGCCCAGTAA*	58	483	A	55	1.5	0.1
HP8 ^a	ACCTAGTGTTCACAAGCAG*	58	CCTCGGACTCAGCGCAGCAA	62	295	A	55	1.5	0.1
<i>HFE</i> gene coding regions [Reference sequence: NM_000410 (GenAtlas)]									
H1 ^d	TACTGGGCATCTCCTGAGC	62	CTAGTTTCGATTTTCCACCCC	61	256	A	55	2	0.1
H2A	^e ACATGGTTAAGGCCTGTTGC*	60	^d TACCCCTTGCTGGTGTGA*	60	298	A	55	2	0.1
H2B	^d TGACCAGCTGTCGTGTTCT*	60	^e CAGCTGTTCCCTCAAGATGCA*	61	257	A	55	2	0.1
H3A ^d	CTTGGGGATGGTGGAAATAG	60	CTCCAGGTAGGCCCTGTTCT	65	279	A	57	1.5	0.1
H3B ^d	CGAGGGCTACTGGAAGTACG	65	CTGCAACCTCCTCCACTCTG	65	280	A	57	1.5	0.1
H4 ^f	TGGCAAGGGTAAACAGATCC*	60	CTCAGGCACCTCCTCAACC*	65	390	A	55	2	0.1
H5	^d GAGAGCCAGGAGCTGAGAAA	62	^e CAGAGGTAAGAGACTTC	58	297	A	55	2	0.1
H6 ^e	TAGTGCCCCAGGCTAAATTG	58	TGAGTCTCTAGTTTGTCTCC	59	202	A	57	1.5	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; dATP, 2'-deoxy-adenosine-5'-triphosphate; dCTP, 2'-deoxycytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dNTP, 2'-deoxynucleotide; dTTP, 2'-deoxy-thymidine-5'-triphosphate; *HFE*, high-iron gene; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; T_m, melting temperature. T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986).
References: ^aThis study; ^dV Human (unpublished data); ^eProf C Camaschella (unpublished data); primers applied by Feder *et al*, 1996 All primers supplied by Inqaba Biotech except * supplied by IDT (Integrated DNA Technologies).

Table 2.3. Oligonucleotide primers designed for the PCR amplification of the *HAMP* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	Ann 2 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
<i>HAMP</i> gene promoter region [Reference sequence: ENSG00000105697 (Ensembl)]										
PP1 ^a	GACGGGGAGGTTTCTAA	56	AGCCTGGGTGACAGAGTGAG	56	274	A	56		1	0.1
PP2 ^a	CATCGGACTGTAGATGTTAGC	60	TCAAGACTAGCCCTGGGCAAC*	62	256	A	57.5		1.5	0.1
PP3 ^a	CACGCCTGGCTAAATTTGTT*	58	CACCACACGTGCATAGGTTT*	62	326	A	55		2	0.1
PP4 ^a	TCAAAGGCTCTGACACTGGG*	62	CCATCAGCATGTCATTCTGC*	60	312	B	59	54	1.5	0.1
PP5 ^a	AAGTGAGTGGAGGAGGCG	62	CTTTGCTCTGTCTCATTTC	58	285	A	55		2	0.1
PP6 ^a	CTGAGGGTGACACAACCCCT	60	AGAGCCACTGGTCAGGCTG	62	291	A	56		1	0.1
<i>HAMP</i> gene coding regions [Reference sequence: NM_021175.2 (GenAtlas)]										
P1 ^d	AGCAAAGGGAGGGGCTCAGACC*	80	TCCCATCCCCTGCTGCCCTGCTAAG*	78	262	C	60		1.5	0.1
P2 ^g	AAACCACCTGGAGAGGAGCA	60	GAAGGAAGGGAATGTGAGCA	60	235	C	55		1.5	0.1
P3 ^g	GCAACAGTGATGCCCTTTCCT*	60	CCAGCCATTTATCCCAAGACC*	61	272	C	55		1.5	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; dATP, 2'-deoxy-adenosine-5'-triphosphate; dCTP, 2'-deoxy-cytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dNTP, 2'-deoxynucleotide; dTTP, 2'-deoxy-thymidine-5'-triphosphate; *HAMP*, hepcidin antimicrobial peptide gene; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; T_m, melting temperature; T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986). References: ^aThis study; ^dV Human (unpublished data); ^gSupplied by Dr A Merryweather Clarke (unpublished data). All primers supplied by Inqaba Biotech except * supplied by IDT (Integrated DNA Technologies).

Table 2.4. Oligonucleotide primers designed for PCR amplification of the *SLC40A1* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	Ann 2 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
SLC40A1 gene promoter region [Reference sequence: ENSG00000138449 (Ensembl)]										
SP1 ^a	GTAGACCTTTGGGGCTCCTG*	64	TGGAGGGTGAGGTGAATGAC*	62	288	C	60		1.5	0.2
SP2 ^a	GGCACAGCAGGATTAACCG	60	AATCCGTCCTGGAGACAGAA	60	289	C	60		1.5	0.1
SP3 ^a	GGGAGAAAGGAATGATGGTGA	60	ATGCCACAGAGGCCGCTTTC	60	302	C	57.5		1	0.2
SP4 ^a	GAAGCCCTGCTATGCAGTCC*	64	GTCAGGTGCTGGAGAAAGC*	64	255	C	60		1	0.2
SP5 ^a	CTGGAGCTTTGCACCTGCGAC*	64	AGACGAGCTCCCGTCAACCT*	60	357	B	58	54	1.5	0.1
SP6 ^a	TGGAACGCGTCGAGGCCGAA*	62	AGCTAACACTGTAGCTGAAG*	58	369	N/O				
SP7 ^a	CTGAGCCTCCCAACCCTT	64	TCACCACAGCATCCTCTCTG*	62	315	C	60		1	0.2
SLC40A1 gene coding regions [Reference sequence: NM_014585 (GenAtlas)]										
S1A ^h	CCAGTCGGAGGTCGCAGG	67	CAGGAGTGC AAGGAACCTGG	62	318	C	60		0.75	0.1
S1B ^h	CCAAAGTCGTCGTTGATGC	60	TTCCTCCAGAACTCGTGATG	60	276	A	55		2	0.1
S2 ^h	TGGATAAGCATTCTGCCCTC	60	AAAGCATGTGACTTGGATG	56	275	A	55		2	0.1
S3 ^h	GATAAGGAAGCAACTTCTCTG	59	CCTGGTTGTTTCTCTCTCTAG	60	339	B	60	55	2	0.1
S4 ^h	GGATAAGAACAGTCTCACTG	58	TTCATCCTTTACCCTACCAG	60	243	B	60	55	2	0.1
S5 ^h	TTAAACTGCCCTTGTGTTAGTG	54	GCCTCATTATCACCACCG	58	278	B	60	55	2	0.1
S6 ^h	TTGTGTAATGGCAGTCTC*	58	CATTTAAGGTCTGAACATGAG*	57	368	C	60		3	0.1
S7A ^h	GCTTTTATTCTACATGTCC	54	CCAGTTATAGCTGATGCTC	58	352	C	60		2	0.1
S7B ^h	GGGTACGCCCTACACTCAG	62	CAGTTGTAATTTTCAGGTATC	54	298	B	60	55	2	0.1
S7C ^h	GAAGATATCCGATCAAGGTTT	59	TTAATGGATTCTCTGAACCTAC	57	259	A	55		2	0.1
S8A ^h	TTGAAATGTATGCCTGTAAAC	55	TTCCTTCCTAACCTCTTTTTC	57	343	C	60		3	0.1
S8B ^h	CCGATTTGCCCAAAATACTC	58	TTTCCATGCCCTCAACATAAGG	59	297	A	55		2	0.1
S8C ^h	GTTTTTACCACAGCTGTGCC*	60	GTCTTCATACTTGAAGAATTTG	55	359	A	55		2	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; dATP, 2'-deoxy-adenosine-5'-triphosphate; dCTP, 2'-deoxy-cytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dNTP, 2'-deoxynucleotide; dTTP, 2'-deoxy-thymidine-5'-triphosphate; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; *SLC40A1*, solute carrier family 40 (iron-regulated transporter) member 1 gene; T_m, melting temperature. T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986). References: ^aThis study; ^hL. Bloem (unpublished data); ⁱprimers applied by Njajjou *et al.* 2001. All primers supplied by Inqaba Biotech except * supplied by IDT (Integrated DNA Technologies).

Table 2.5. Oligonucleotide primers designed for PCR amplification of the *CYBRD1* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
<i>CYBRD1</i> gene promoter region [Reference sequence: ENSG00000071967 (Ensembl)]									
BP1 ^a	CACACTAAACGGCAAGTCCA	60	GTCCCCATGAGGTGCTCTCAC	64	255	A	57	1.5	0.1
BP2 ^a	CAGTGCAGTGGTGCATCAT	62	GTGAACCCTGTCTCTACTG	60	310	A	56	1.5	0.1
BP3 ^a	GCTCAAGTGATCCTCCTCC	62	GCCTCCTTGCAGGCTATAAC	62	275	A	57	1.5	0.1
BP4 ^a	CTGCGCCTGGCCTTACTGT	64	GAGAACATGTTACATGCATG	56	300	A	55	1.5	0.1
BP5 ^a	AGTTACAGGAGAGCTATG	60	TGCACCAGCCAGAACTACAG	62	294	A	56	1.5	0.1
BP6 ^a	CTGTACACTGGCCAGAACC	64	AGCGGACAGAACTACTCT	62	309	A	56	1.5	0.1
BP7 ^a	GTAGGAGTGTGACTTAGG	60	GCTGGAGAATTGGCTGTCTC	62	312	A	58	1.5	0.1
BP8 ^a	GGTCCATTGAGTCAGTGAGG	62	CACATCCCGTCTCGTCTC	64	245	A	58	1.5	0.1
BP9 ^a	CTACCCCAACGGATCCCTCTC	64	CTGGCCCCAACTCAGAAATG	62	276	A	58	1.5	0.1
BP10 ^a	AAGCCCTCTCGGAGCTTGG	62	GCGAAGATCACCGACAGGAA	62	372	A	55	1.5	0.1
<i>CYBRD1</i> gene coding regions [Reference sequence: NM_024843 (GenAtlas)]									
Cy1 ^b	GAGACAGCCCCAAGAAGTCG [#]	65	TTCACGGAGGACCCTCTGCC [#]	67	378	D	60.5	2	0.1
Cy2 ^b	CCAGTGTGCAAACTGTC	58	CATTACAGTCTCTGAATTG	54	346	D	51.1	2	0.1
Cy3 ^b	TTGTCAATATTACATATTGC [#]	54	CATTTTCCCAGTGAACAAGTA [#]	57	318	D	53.8	2	0.1
Cy4A ^b	GCATGTTGCTGTATCATCCTGT [#]	61	AGAGTAGGCTGGCATGGAAC [#]	62	254	D	57	2	0.1
Cy4B ^b	AAATGGAGGCACCTGAACAGG [#]	60	AGGAGAAGCAAAACTGTAGAGC [#]	61	217	D	57	2	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; *CYBRD1*, cytochrome b reductase 1 gene; dATP, 2'-deoxyadenosine-5'-triphosphate; dCTP, 2'-deoxy-cytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dNTP, 2'-deoxynucleotide; dTTP, 2'-deoxy-thymidine-5'-triphosphate; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; T_m, melting temperature. T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986). References: ^aThis study; ^bF Booley (unpublished data). All primers supplied by IDT (Integrated DNA Technologies) except [#] supplied by Inqaba Biotech.

Table 2.6. Oligonucleotide primers designed for PCR amplification of *HJV* gene promoter and coding regions

Primer set name	Forward Primer (5'-3')	T _m (°C)	Reverse Primer (5'-3')	T _m (°C)	PCR Product Size (bp)	PCR cycle	Ann 1 (°C)	MgCl ₂ (mM)	Each dNTP [dATP, dTTP, dCTP, dGTP] (mM)
<i>HJV</i> gene promoter region [Reference sequence: ENS00000168509 (Ensembl)]									
JP1 ^a	TGGCTTAGTGATTGTTGG*	58	GCAAGGAGAGCCAGTCTCAG*	64	332	A	55	1.5	0.1
JP2 ^a	CATATTTCTCCCATGCTGGA	58	ACCGATTCCAAATACGGTC*	56	383			1.5	0.1
JP3 ^a	GAAAGCAGACTGGTGATGGC	62	CTGCAACCAAGGGATTAGC	60	258	A	55	1.5	0.1
JP4 ^a	TGACCGTATTTGGAATCGGTC	62	TCAGCACTTTGGAGCTGAG	62	259	A	60	1.5	0.1
JP5 ^a	CATAGCTCACTGCAGCCTC	60	TCCTTCTCCGAGATGAG*	58	252	N/O		1.5	0.1
JP6 ^a	GATGGAAGGATTCATTGAGGC*	62	CAGCAATTCATCTTCTCTCC*	62	323	A	57	1.5	0.1
JP7 ^a	CCTTCTCTCCAAGCAAATC	56	CCATAGCAGAGGTAGTTCA	56	263	A	55	1.5	0.1
JP8 ^a	CTTCTCTGGACAGCTGGCT	60	GTCACCTGCCTACTCCAGGC	62	260	A	57	1.5	0.1
JP9 ^a	CAGCATAGCAGGGATGAAC	58	AGTCCAAAATCCAGGAACG	58	286	A	55	1.5	0.1
JP10 ^a	TTCTAATCCTCTAACCCTCC	58	GACTTACTGTTCCAGAGGTC	62	332	A	55	1.5	0.1
<i>HJV</i> gene coding regions [Reference sequence: ENS00000168509 (Ensembl)]									
J1 ^b	TCTGGCCAGCCATATACTCC	62	CAGCATTGGACGAGAGA	54	293	D	58	1.5	0.1
J2 ^b	CACTCCACATTATCCTTACC	58	ATGCCACCCCTACATAGC	62	284	D	56	2	0.1
J3A ^b	ACACTCCGATAGACAGAGG	62	TC TTCGATGCCATGTACCG	60	298	D	56	2	0.1
J3B ^b	TAGAGGTGGGGTTTCATCAG	62	CGGCCCTTCATAGTCACAAGG	62	300	D	58	2	0.1
J3C ^b	GACCTGATGATCCAGCACAA	60	TGGCTTGGACAAAAGAGGAAG	60	287	D	56	2	0.1
J3D ^b	CCGGACCCCTTGTGACTATGA	62	GTGCCCTGGAAGAATCTC	60	279	D	58	2	0.1
J4A ^b	TCAAGGATTGAGGGCCATAG	60	TGGATCTCCACATGGTTCC	60	300	D	56	2	0.1
J4B ^b	GGTGGATAATCTTCTGTAGC	61	CGACGATTGCGCTCTGAT	60	288	D	56	2	0.1
J4C ^b	GCTCTCCTTCTCCATCAAGG	62	CTGAGCTGCCACGGTAAAGT	62	256	D	58	2	0.1
J4D ^b	GGGCTTCCAGTGGAAAGTGC	65	CCCCTTACTGAATGCAAAGC	60	238	D	58	2	0.1
J4E ^b	CATCTCTTCCCTCAGATGC	62	GATCCGGAATGCAGTAACT	60	300	D	56	2	0.1
J4F ^b	AAGCAGGGCCCTAGGAGACAC	65	TGCTTTCAGCTCTTGCCTCT	60	283	D	58	2	0.1
J4G ^b	CTGCATCCGGATCTCTGTG	62	TTTTGAATCAAGAAAGCAGAACA	56	291	D	56	2	0.1
J4H ^b	TGTGTGTGTAAGGTAATGTTCTGC	61	CTGATACTCCGAGCCCTCTTTC	65	261	D	58	2	0.1

Abbreviations: 3', 3-prime end; 5', 5-prime end; Ann, annealing temperature; bp, base pair; °C, degrees Celsius; dATP, 2'-deoxy-adenosine-5'-triphosphate; dCTP, 2'-deoxy-cytidine-5'-triphosphate; dGTP, 2'-deoxy-guanosine-5'-triphosphate; dNTP, 2'-deoxy-nucleotide; dTTP, 2'-deoxy-thymidine-5'-triphosphate; *HJV*, hemojuvelin gene; MgCl₂, magnesium chloride; mM, millimolar; N/O, not optimised; PCR, polymerase chain reaction; T_m, melting temperature; T_m = 2(nA+nT) + 4(nG+nC) (Thein and Wallace 1986).
References: ^aThis study; ^bF Booley (unpublished data). All primers provided by Inqaba Biotech except * supplied by IDT (Integrated DNA Technologies).

2.2.4 Agarose Gel Electrophoresis

Successful amplification of PCR products was tested on a 2% (w/v) horizontal agarose gel [consisting of 4 g agarose, 1 × Tris-Borate/EDTA (TBE) (90 mM Tris-HCl (pH 8.0), 90 mM boric acid (H₃BO₃) and 1 mM EDTA) and 0.01% (v/v) ethidium bromide (EtBr)]. The PCR product (5 µl) was mixed with Cresol red loading buffer (5 µl) [consisting of 0.02% (w/v) cresol red and 0.34% (w/v) sucrose) and loaded into the wells of the agarose gel. The products were resolved at 120 V for 1 hour in 1 × TBE buffer solution. A 100 base pair (bp) marker (Fermentas) established amplification of the correct fragment size. The DNA was visualised by ultraviolet light transillumination on the Multigenius Bio Imaging System (Syngene, Cambridge, United Kingdom (UK)).

2.2.5 Heteroduplex Single-Strand Conformation Polymorphism (HEX-SSCP) Analysis

The successfully amplified DNA fragments were subjected to HEX-SSCP analysis (Kotze *et al*, 1995) performed on a Hoefer vertical gel apparatus. The fragments were electrophoresed on a 12% (w/v) polyacrylamide (PAA) gel supplemented with urea [(NH₂)₂CO] [gel consisting of 7.5% (w/v) urea, 1.5 × TBE buffer (135 mM Tris-HCl (pH 8.0), 135 mM boric acid and 1.5 mM EDTA), 12% (w/v) PAA (1%C of a 40% stock [99 acrylamide (AA):1 bisacrylamide (BAA)], 0.1% (w/v) ammonium persulphate (APS) and 0.1 % (v/v) TEMED.

Gels were cast at room temperature and allowed to completely polymerise. Subsequently, the gels were placed into the Hoefer electrophoresis tank, which contained 1 × TBE buffer. The upper buffer chamber was filled with fresh 1.5 × TBE buffer. A volume of 15 µl of bromophenol blue loading buffer [consisting of 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 95% (v/v) formamide (de-ionised) and 20 mM EDTA] was added to the PCR products (20 µl). The solution was then heat denatured at 95°C for 10 minutes and immediately placed on ice. Approximately 15 µl of the denatured PCR product was loaded on the gel and electrophoresed at 4°C at 250 V for 18 hours.

Following electrophoresis, the gels were dismantled and the DNA stained in a 0.01% (v/v) EtBr solution for 10 minutes. This was followed by 3 minutes of destaining in ddH₂O. DNA fragments were visualised by ultraviolet light transillumination and photographed using the Multigenius Bio Imaging System (Syngene, Cambridge, UK).

2.2.6 Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP analysis was performed for the *CYBRD1* variant S266N and the *HMOX1* variant IVS2+4T→C as successful genotyping of these two variants was not possible using the HEX-SSCP images.

The S266N variant in exon 4 of the *CYBRD1* gene was digested with *TspRI* (recognition site 5' CAG↓TG 3', New England Biolabs) to improve genotyping of this variant. The *TspRI* recognition site is abolished by the presence of this variant. In the absence of the variant two fragments of 87 bp and 130 bp result. Digestion of the variant DNA fragment produces three fragments of 217 bp, 130 bp and 87 bp in the heterozygous state and 1 fragment of 217 bp in the homozygous state.

RFLP analysis was also used to genotype the IVS2+4T→C variant in the *HMOX1* gene. Exon 2 of the *HMOX1* gene was digested with *HpyCH41V* (recognition site 5' A↓CGT 3', New England Biolabs). This restriction enzyme is an isoschizomer of *MaeII*. This variant creates an *HpyCH41V* recognition site within the exon 2 fragment. In the absence of the variant the DNA fragment remains uncut and produces a single 236 bp fragment. Digestion of the variant DNA fragment produces three fragments of 236 bp, 163 bp and 73 bp in the heterozygous state and two fragments of 163 bp and 73 bp in the homozygous state.

All RFLP reactions were performed in a final volume of 20 µl that contained 10 µl of the relevant PCR product, 1 × buffer and 2 U of the applicable restriction enzyme. PCR products were incubated in a water bath for 16 hours to allow complete digestion. Incubation temperatures and buffer components for each restriction enzyme are supplied in Table 2.7.

Electrophoresis of all of the digested PCR products was performed on a 2% (w/v) agarose gel (see Section 2.2.4). A volume of 10 µl of each digested PCR product was mixed with 10 µl Cresol red loading buffer and loaded into the wells of the agarose gel. The products were resolved at 100 V for 90 minutes in 1 × TBE buffer solution. The sizes of the digested DNA fragments were verified by loading a 100 bp DNA marker (Fermentas) along with the samples. The DNA fragments were visualised by ultraviolet light transillumination on the Multigenius Bio Imaging System (Syngene, Cambridge, United Kingdom (UK)) for successful genotyping.

Table 2.7. Table outlining restriction enzyme buffers and water bath incubation temperatures

Gene	Variant	Restriction Enzyme	NEBuffer	Buffer Components	Incubation Temperature (°C)
<i>HMOX1</i>	IVS2+4T→C	<i>HpyCH41V</i>	1	10 mM Bis Tris Propane-HCl 10 mM MgCl ₂ 1 mM DTT pH 7.0 at 25°C	37
<i>CYBRD1</i>	S266N	<i>TspRI</i>	4	50 mM KAc, 20 mM TA, 10 mM MgAc, 1 mM DTT pH 7.9 at 25°C Supplemented with 100 µg/ml BSA	65

Abbreviations: µg/ml, microgram per millilitre; BSA, bovine serum albumin; °C, degrees Celsius; *CYBRD1*, cytochrome b reductase 1 gene; DTT, dithiothreitol; HCl, hydrochloric acid; *HMOX1*, haem oxygenase-1 gene; *HpyCH41V*, *Escherichia coli* strain carrying the cloned *HpyCH41* gene from *Helicobacter pylori* CH4; IVS, intervening sequence; KAc, potassium acetate; MgAc, magnesium acetate; MgCl₂, magnesium chloride; mM, millimolar; N, asparagine; NEBuffer, New England Biolabs buffer; S, serine; TA, tris-acetate; *TspRI*, *Escherichia coli* strain carrying the cloned *TspRI* gene from *Thermus* species R.

2.2.7 Semi-automated DNA Sequencing Analysis

Samples showing aberrant banding patterns upon HEX-SSCP analysis were subjected to bi-directional semi-automated DNA sequencing. The PCR products were purified prior to sequencing using the GenElute™ PCR Clean-Up Kit (Sigma).

The clean-up protocol was initiated with the insertion of a GenElute Miniprep Binding Column into the collection tube and addition of a volume of 500 µl of Column Preparation Solution to each column. Each tube was then centrifuged at 11 200 rpm (Centrifuge 5415D, Eppendorf) for 1 minute. The eluate was subsequently discarded. The PCR product was prepared by adding 5 volumes of Binding Solution to 1 volume of the PCR product. Following mixing, the solution was centrifuged for 1 minute at 13 000 rpm. After discarding the flow-through, 500 µl of Wash Solution was added to the column and it was centrifuged at 13 000 rpm for 3 minutes. The column was transferred to a clean 2 ml collection tube and 50 µl of ddH₂O was applied to the centre of each column. The tube was incubated at room

temperature for 1 minute before the DNA was eluted by centrifugation at 13 000 rpm for 1 minute. The purified DNA was subsequently stored at 4°C. The constituents of the GenElute™ PCR Clean-Up Kit (Sigma) Column Preparation, Binding and Wash Solutions were not made available by the manufacturer.

Cycle sequencing was performed on a GeneAmp®PCR system 2700 thermocycler. Each reaction contained 3.3 ng of the relevant primer (Tables 2.1-2.6), 1 µl termination ready reaction mix [BigDye® Terminator v3.1 cycle sequence kit (Applied Biosystems)] and 9.9 ng of the purified PCR product.

The cycle program consisted of an initial denaturation step of 10 seconds at 96°C followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 60°C for 4 minutes. The samples were subsequently sent to a sequencing facility (Central Analytical Facility, Stellenbosch University) where they were loaded onto an ABI PRISM 3130X1 Genetic Analyser (Applied Biosystems) automated sequencer. Analysis of all resulting sequences was performed by a) visually examining the electropherograms and b) alignment of sequences with the reference sequence (accession numbers of reference sequences are listed in Tables 2.1-2.6) using BioEdit Sequence Alignment Editor v7.0.1 (Hall, 1999).

CHAPTER THREE

RESULTS AND DISCUSSION

Mutation analysis of six genes in an Indian family with Hereditary Haemochromatosis

Abstract

Hereditary haemochromatosis (HH) is a very common disease in individuals of northern European descent. The majority of patients are homozygous for the C282Y mutation in the *HFE* gene or compound heterozygotes for the C282Y and H63D mutations. Although prevalent in Caucasians, the disease is rare in Asians and Africans. The present study attempted to elucidate the gene variant, or variants, causing HH in an Asian Indian family as well as potential modifiers of the HH phenotype. Mutation analysis of the promoters and coding regions of six iron regulatory genes including *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* and *HJV*, was performed. Heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis and bi-directional semi-automated DNA sequencing analysis were employed to identify variants associated with HH in this family. Twenty four previously identified and five novel variants (*HFE*: 5'UTR-840T→G; *CYBRD1*: 5'UTR-1813C→T, 5'UTR-1452T→C, 5'UTR-1272T→C; and *HJV*: 5'UTR-534G→T, 5'UTR-530G→T) as well as one known *SLC40A1* repeat and two novel 5'UTR repeats (*CYBRD1*: [G(T)₈G(T)₆G(T)_nG(T)₉] where n represents zero or six repeats and *HJV*: (AAGG) presenting with 11 to 13 repeats) were identified in the Indian family. A propitious previously identified *HAMP* variant (5'UTR-335G→T) seems to be responsible for the iron overload phenotype observed in the two Indian probands. Variants identified in the *HMOX1* and *HFE* genes may be modifying the effect of the *HAMP* promoter variant.

Introduction

Hereditary haemochromatosis (HH) is a common autosomal recessive disorder resulting from the disruption of iron absorption. The majority of patients have a mutation in the high-iron (*HFE*) gene, with C282Y homozygosity accounting for more than 80% of HH cases in Caucasians of northern European descent (Feder *et al*, 1996). The C282Y homozygous genotype results in the most severe form of HH, referred to as Type 1, and is presented by approximately five out of every 1000 individuals of northern European descent. However, the penetrance of this variant seems to be incomplete. The proportion of HH patients presenting

with the homozygous C282Y variant genotype differs in distinct populations and the variant allele is not present in the Asian and African populations (Merryweather-Clarke *et al*, 1997; Roth *et al*, 1997). Other types of haemochromatosis not associated with the *HFE* gene include Type 2 haemochromatosis resulting from mutations in the *HAMP* and *HJV* genes (Roetto *et al*, 2003; Papanikolaou *et al*, 2004), Type 3 resulting from mutations in the *TFR2* gene (Camaschella *et al*, 2000), Type 4 associated with *SLC40A1* mutations (Njajou *et al*, 2001) and Type 5 resulting from mutations in the H-ferritin gene (Kato *et al*, 2001).

Although HH Types 1, 2 and 3 are distinct diseases, they do share similar disease phenotypes. Researchers have discovered that in cases of juvenile haemochromatosis where patients display mutations in the *HJV* gene, almost no urinary hepcidin is detected (Papanikolaou *et al*, 2004). This may indicate that *HJV* and *HAMP* function in the same pathways and that *HJV* may regulate *HAMP* expression. Babitt *et al* (2006) revealed that *HJV* regulates hepcidin expression by acting as a cofactor in the BMP signalling cascade. Hepcidin expression is also diminished in patients with haemochromatosis due to mutations in *HFE* or *TFR2*. This implicates *HFE* and *TFR2* in the regulation of hepcidin expression and how these two proteins regulate hepcidin needs to be investigated further (reviewed by Ganz and Nemeth, 2006).

HH is predicted to be rare in the Indian subcontinent. In various studies the frequency of the C282Y and H63D variants has been determined, but these variants are not associated with iron overload in India (Garewal *et al*, 2005; Dhillon *et al*, 2007). It is believed that haemochromatosis in India is of the non-*HFE* type but the gene causing the HH phenotype has not been elucidated as yet.

Mutation analysis of six genes namely, the haem oxygenase-1 (*HMOX1*) gene, high-iron (*HFE*) gene, hepcidin antimicrobial peptide (*HAMP*) gene, solute-carrier family 40 (iron-regulated transporter) member 1 (*SLC40A1*) gene, cytochrome b reductase 1 (*CYBRD1*) gene and hemojuvelin (*HJV*) gene, was performed in an attempt to elucidate the causative gene variant or variants associated with HH in this Indian family.

Materials and Methods

Information provided for all study participants, detailed methodologies employed and statistical analysis performed are as outlined in Chapter 2.

Results

3.1 Body Iron Status

The TS% and SF levels for the two probands (at diagnosis) are provided in Table 3.1. A TS% exceeding 45% and/or a SF level of more than 200 $\mu\text{g/l}$ in females and 300 $\mu\text{g/l}$ in males were indicative of iron overload. The TS% and SF levels for 20 of the phenotypically unaffected family members are also shown in Table 3.1. For one of the extended family members (III:19) only the TS% was provided. No iron parameters were available for three of the family members (III:3, III:5 and III:17). The iron parameters of the extended family members are provided as of date of study.

Table 3.1. Characteristics and iron indices of probands and unaffected family members

Patient	Sex	Age	Transferrin Saturation (%)	Serum Ferritin (µg/l)
Proband 1*	Male	64	100	5220
Proband 2*	Female	61	58	595
II:2	Male	63	15	29
II:4	Female	60	30	191
II:5	Male	57	29	37
II:6	Female	52	18	25
II:7	Male	50	22	277
III:1	Male	42	30	196
III:2	Female	36	24	100
III:3	Male	23	unknown	unknown
III:4	Female	42	12	62
III:5	Male	36	unknown	unknown
III:7	Female	43	13	15
III:8	Female	41	26	118
III:9	Male	40	40	137
III:10	Female	39	33	213
III:12	Male	34	29	264
III:14	Female	34	23	115
III:16	Female	27	34	108
III:17	Male	29	unknown	unknown
III:19	Male	19	22	unknown
III:20	Female	19	44	91
III:21	Female	10	22	78
III:22	Female	16	31	76
III:23	Female	40	17	57

* Iron parameters are given for the probands at diagnosis.

3.2 Mutation Analysis

Mutation analysis of the six genes namely, *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* and *HJV*, in the two probands revealed several variations on HEX-SSCP analysis. Twenty four previously described single nucleotide substitutions were identified as well as a previously described repeat. In addition to these known variants, five novel single nucleotide substitutions and two repeats were identified in the two probands. All variants are shown in Table 3.2. The position of each variant and repeat identified is indicated on the reference sequence of each gene in Appendix 2. The genotypes of the two Indian probands for each variant are shown in Appendix 3.

The polymerase chain reaction (PCR) could not be optimised for the amplification of the *SLC40A1* promoter fragment 6 nor the *CYBRD1* promoter fragment 2 (see primer Tables 2.4 and 2.5). The *SLC40A1* promoter region that was spanned by fragment 6 was however amplified using the adjacent forward and reverse primers namely, SP5 forward and SP7 reverse (see Table 2.4). The resulting 840 bp fragment could not be analysed using HEX-SSCP analysis so direct sequencing analysis was performed on this fragment. Likewise, the *CYBRD1* promoter region spanned by the BP2 primers was amplified using the adjacent BP1 forward and BP3 reverse primers (see Table 2.5). The resulting 747 bp fragment was subsequently subjected to direct sequencing analysis. Optimisation of PCR amplification for the *HMOX1* promoter fragment 5 (Table 2.1), *HAMP* promoter fragment 2 (Table 2.3), and *HJV* promoter fragments 2 and 5 (Table 2.6) was unsuccessful and therefore mutation analysis of these fragments was incomplete.

After initially being identified in the probands, all variants were screened for in the 23 extended family members. The genotype of the family members for each variant is shown in Appendix 3. For some variants amplification of the respective PCR fragment was unsuccessful and therefore genotyping of that variant is incomplete.

Table 3.2. Variants initially identified in two probands of an Indian family and subsequently identified in the extended family members

Gene	5'UTR/Exon/Intron	Variant	Reference
<i>HMOX1</i>	5'UTR	5'UTR-495A→T	rs2071746*
	2	IVS2+4T→C	rs17885925*
	2	IVS2-19C→T	rs7879606*
<i>HFE</i>	5'UTR	5'UTR-1206C→G	rs1800702*
	5'UTR	5'UTR-467G→C	rs2794720*
	5'UTR	5'UTR-840T→G	This study
	2	H63D (g.189C→G)	Feder <i>et al</i> , 1996
<i>HAMP</i>	5'UTR	5'UTR-335G→T	rs3817623*
<i>SLC40A1</i>	5'UTR	5'UTR-1355G→C	rs3811621*
	5'UTR	5'UTR-750G→A	rs13015236*
	5'UTR	5'UTR-593C→T	rs12693542*
	5'UTR	5'UTR-501T→C	rs6728200*
	1	(CGG) ₈ [#]	Lee <i>et al</i> , 2001
	1	(CGG) ₇ [#]	Lee <i>et al</i> , 2001
	6	V221 (g.663T→C)	Devalia <i>et al</i> 2002
<i>CYBRD1</i>	5'UTR	5'UTR-1844C→G	rs7585974*
	5'UTR	5'UTR-1834G→A	rs7586174*
	5'UTR	5'UTR-1813C→T	rs12692965
	5'UTR	5'UTR-1459T→C	rs10199858*
	5'UTR	5'UTR-1452T→C	This study
	5'UTR	5'UTR-1272T→C	This study
	5'UTR	5'UTR-624G→A	rs884408*
	5'UTR	5'UTR-238A→G	rs868106*
	5'UTR	5'UTR-167C→G	rs2356782*
	5'UTR	5'UTR-163G→A	rs3731976*
	5'UTR	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉ [#]	This study
	5'UTR	G(T) ₈ G(T) ₆ G(T) ₉ [#]	This study
	2	IVS2+8T→C	Zaahl <i>et al</i> 2004
	4	S266N (g.797G→A)	McKie <i>et al</i> 2001
<i>HJV</i>	5'UTR	5'UTR-1406C→A	rs1830823*
	5'UTR	5'UTR-542A→G	rs10910811*
	5'UTR	5'UTR-534G→T	This study
	5'UTR	5'UTR-530G→T	This study
	5'UTR	5'UTR(AAGG) ₁₁ [#]	This study
	5'UTR	5'UTR(AAGG) ₁₂ [#]	This study
	5'UTR	5'UTR(AAGG) ₁₃ [#]	This study

Abbreviations: 5'UTR, 5-prime untranslated region; *CYBRD1*, cytochrome b reductase 1 gene; *HAMP*, hepcidin antimicrobial peptide gene; *HFE*, high-iron gene; *HJV*, hemojuvelin gene; *HMOX1*, haem oxygenase-1 gene; IVS, intervening sequence; N, asparagine; S, serine; *SLC40A1*, solute-carrier family 40 (iron-regulated transporter) member 1 gene; V, valine. [#]position of repeats indicated in Appendix 2; *refSNP ID (HapMap) available online at <http://www.hapmap.org>.

Haplotype analysis was performed to identify any genetic modifiers contributing to the iron overload phenotype in this family. The Cyrillic 2.01 program (Cherwell Scientific Publishing Ltd, Magdalen Centre, Oxford Science Park, Oxford, UK) was employed to perform haplotype analysis for each of the genes under investigation. The variants identified in each of the genes were used as markers to construct the haplotypes. In each case, the wild type allele was denoted with a 1 and the variant allele was denoted with a 2. The length of the repeat represented the different allelic forms of the *SLC40A1* and *HJV* repeats identified. The genotypes of individuals for the *CYBRDI* repeat were represented with a 1 for allele 1 (G(T)₈G(T)₆G(T)₆G(T)₉) and 2 for allele 2 (G(T)₈G(T)₆G(T)₉) of the repeat. The genotypes of some individuals were not clearly distinguishable following HEX-SSCP or DNA sequencing analysis. Furthermore, the genotypes of some individuals were ambiguous in that they did not conform to the haplotypes presented in the Indian family. This ambiguity could not be explained by non-paternity or recombination. The genotypes of these individuals are denoted with question marks in the pedigree. These individuals will be re-analysed and their genotypes clarified employing semi-automated DNA sequencing analysis. Appendix 3 outlines more accurately why the genotypes of these individuals were omitted from the haplotype analysis.

3.2.1 *HMOX1* gene

Mutation analysis of the *HMOX1* coding region and promoter revealed a previously described 5'UTR variant (5'UTR-495A→T) as well as two known intronic variants (IVS2+4T→C and IVS2-19C→T).

A single A to T nucleotide substitution was observed in the promoter region of the *HMOX1* gene 495 nucleotides upstream from the translation initiation site (5'UTR-495A→T) (HapMap; refSNP ID: rs2071746). This previously described variant was observed in the heterozygous state in both probands. Further analysis revealed eight (34.8%) homozygous variant and seven (30.4%) heterozygous family members.

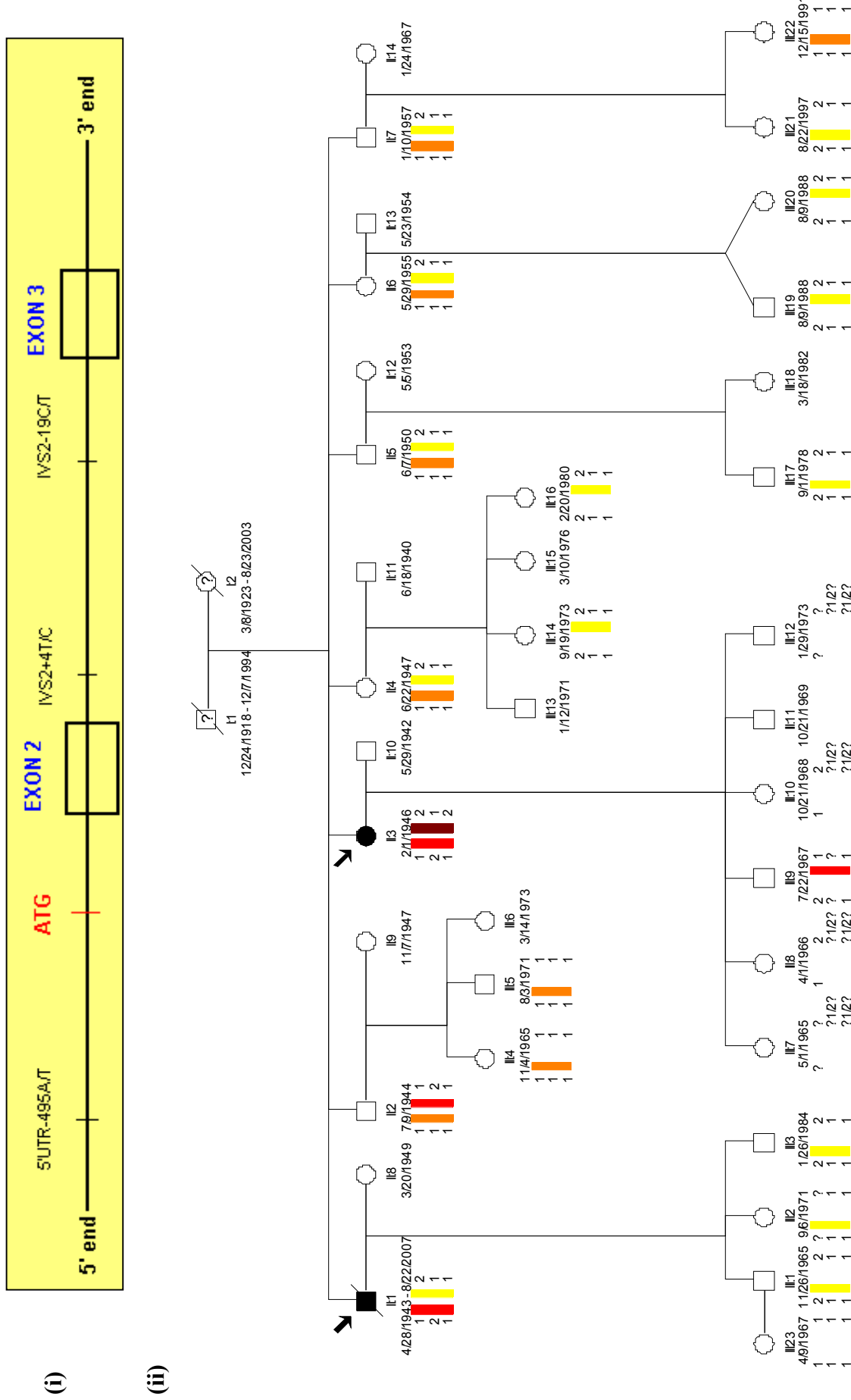
HEX-SSCP and sequencing analysis of exon 2 revealed two previously identified intronic variants. The first, a T to C transition, occurs four nucleotides from the end of exon 2 (IVS2+4T→C) (HapMap; refSNP ID: rs17885925). This variant was identified in the heterozygous state in both of the probands. The variant allele creates a *MaeII* recognition site

(5' A↓CGT 3'). RFLP analysis using the *MaeII* isoschizomer *HpyCH41V* was used for genotyping of the extended family members. The wild type sequence produced a 236 bp fragment while the heterozygous genotype produced three fragments of 236 bp, 163 bp and 73 bp, respectively. The 73 bp fragment was not visible on the 2% (w/v) horizontal agarose gels but genotyping of this variant was still possible using the 236 bp and 163 bp fragments. Five extended family members (21.7%) were heterozygous for this variant while 17 (73.9%) presented with the homozygous wild type genotype. The homozygous state of this variant was not observed.

The second previously described non-coding variant was a C to T transition located in intron 2, 19 nucleotides upstream from exon 3 (IVS2-19C→T) (HapMap; refSNP ID: rs7879606). Proband 2 was heterozygous for this variant while proband 1 was homozygous wild type. Nineteen (82.6%) of the extended family members were homozygous wild type for this variant and four (17.4%) were heterozygous. The homozygous form of this variant was not observed.

Annotation of the *HMOX1* gene variants and the haplotypes constructed for the three variants in the *HMOX1* gene are depicted in Figure 3.1.

Figure 3.1. Haplotypes constructed for the *HMOX1* gene



Legend to Figure 3.1.

Schematic representation of haplotypes constructed for the *HMOX1* gene variants. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the positions of the variants. ii) Pedigree with constructed *HMOX1* haplotypes provided for each individual. Haplotypes are constructed in the order that the gene is annotated. Red, haplotype 1; yellow, haplotype 2; brown, haplotype 3; orange, haplotype 4. Abbreviations: 3', 3-prime end; 5', 5-prime end; ATG, translation initiation site; *HMOX1*, haem oxygenase-1 gene; IVS, intervening sequence; UTR, untranslated region.

3.2.3 HFE gene

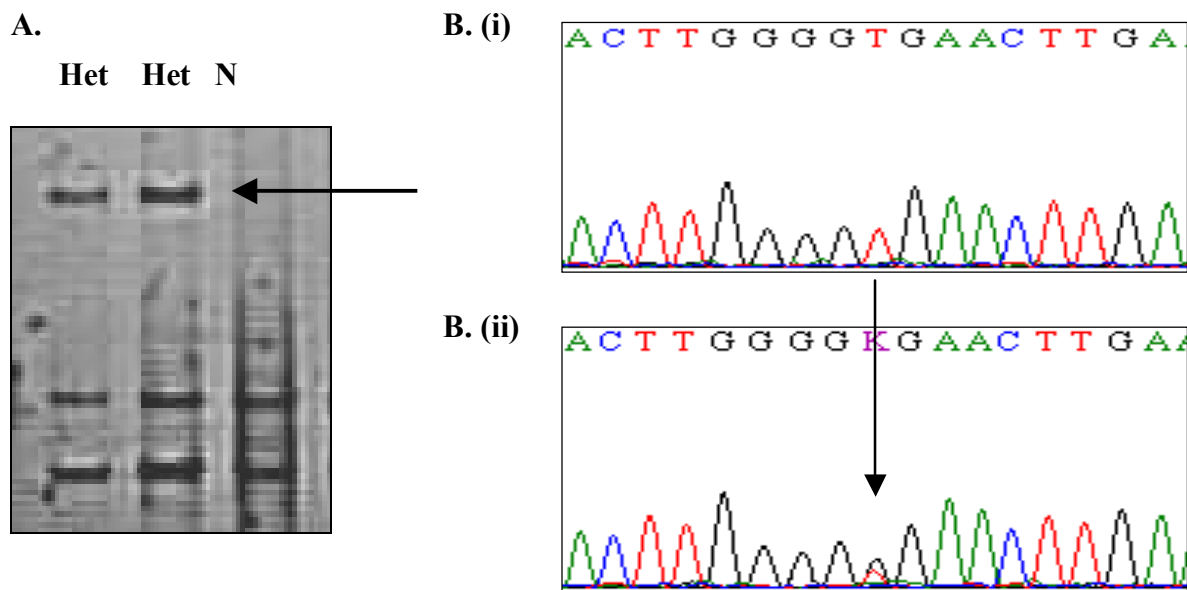
Mutation analysis of the *HFE* gene revealed three previously described variants (5'UTR-1206C→G, 5'UTR-467G→C and H63D) and a single novel variant (5'UTR-840T→G) in the *HFE* gene. The common C282Y *HFE* variant was absent from both of the probands and all family members.

HEX-SSCP analysis and bi-directional semi-automated DNA sequencing of promoter fragment 1 (Table 2.2) identified a known C to G transversion 1206 nucleotides upstream of the translation initiation site (ATG), 5'UTR-1206C→G, (HapMap; refSNP ID: rs1800702). This variant was initially identified in the heterozygous state in both probands. Subsequent genotyping of the extended family members revealed nine heterozygous individuals (39.1%) and two individuals (8.7%) who were homozygous for the variant allele.

A previously described G to C transversion was identified 467 nucleotides upstream of the initiating ATG (5'UTR-467G→C) (HapMap; refSNP ID: rs2794720) in fragment 7 of the promoter (Table 2.2). Both probands were heterozygous for this variant. Analysis in the family members revealed eight heterozygous individuals (34.8%) and two (8.7%) individuals who were homozygous for the variant allele.

A novel variant was identified in the 5'UTR of the *HFE* gene. This variant was observed following HEX-SSCP and sequencing analysis of fragment 3 of the promoter (Table 2.2). The variant is a T to G transversion at nucleotide position 840 upstream from the initiating ATG (5'UTR-840T→G). The HEX-SSCP gel and sequencing electropherograms of this variant are depicted in Figure 3.2. Both probands were heterozygous for this variant as well as three (13%) family members. The homozygous state of this variant was not observed.

Figure 3.2. Schematic representation of the novel 5'UTR-840T→G variant in the *HFE* promoter



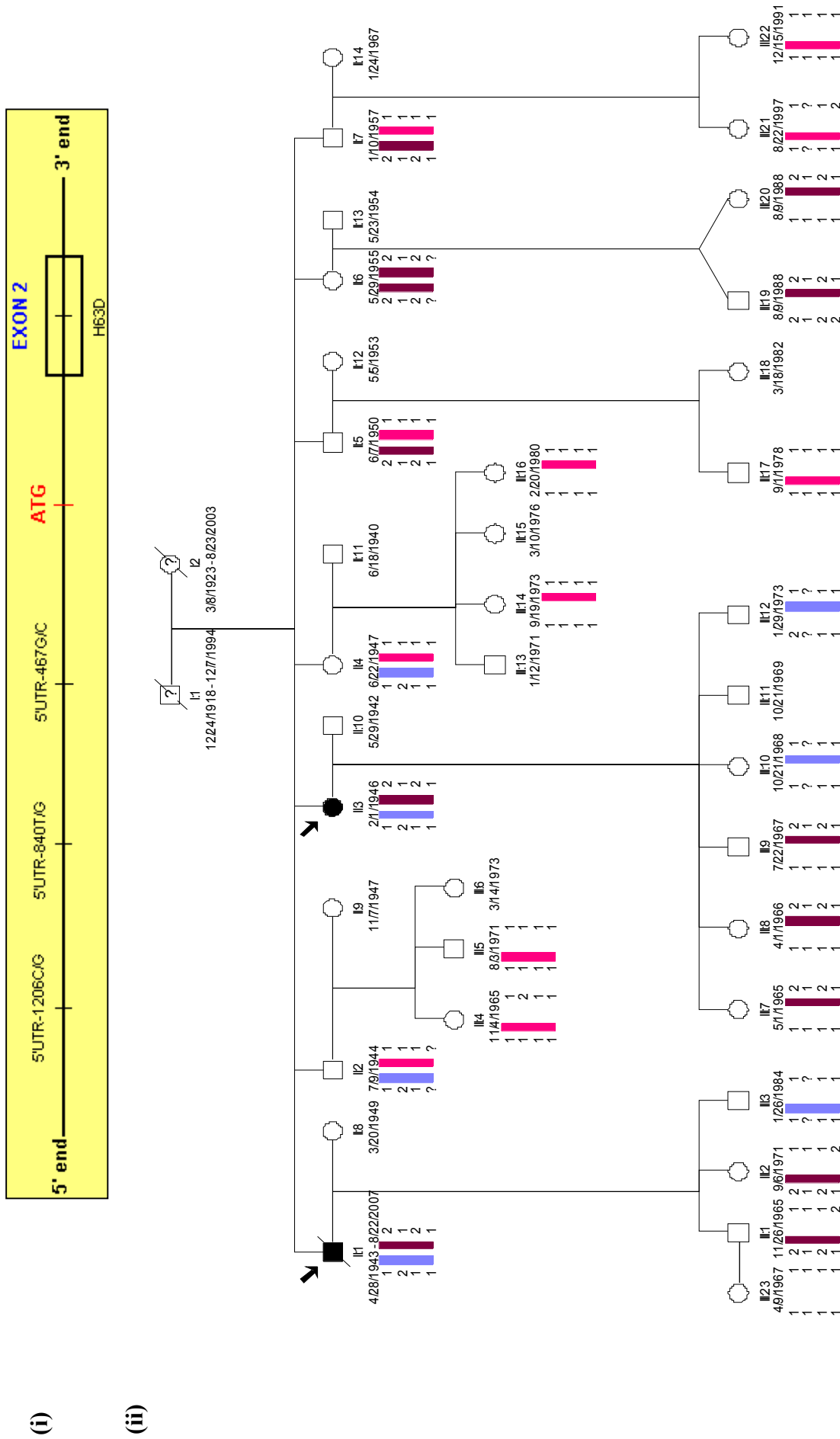
Legend to Figure 3.2.

A. HEX-SSCP gel stained with EtBr and visualised using ultraviolet light transillumination. Arrow indicates aberrant band, Het = heterozygous banding pattern, N = homozygous wild type banding pattern. **B.** Sequencing electropherograms indicating (i) the wild type sequence and (ii) the 5'UTR-840T→G variant. Arrow indicates the point of variation, *green* adenine or A, *blue* cytosine or C, *black* guanine or G, *red* thymine or T.

A missense mutation was observed in exon two of the *HFE* gene. The mutation is a previously described C to G transversion at nucleotide position 189 that results in the replacement of histidine with aspartic acid at amino acid position 63 (H63D) (Feder *et al*, 1996). This mutation was not present in the two probands but was identified in the heterozygous state in four (17.4%) family members.

The *HFE* gene annotation, as well as the haplotypes constructed for the four variants identified in the *HFE* gene, are shown in Figure 3.3.

Figure 3.3. Haplotypes constructed for the *HFE* gene



Legend to Figure 3.3.

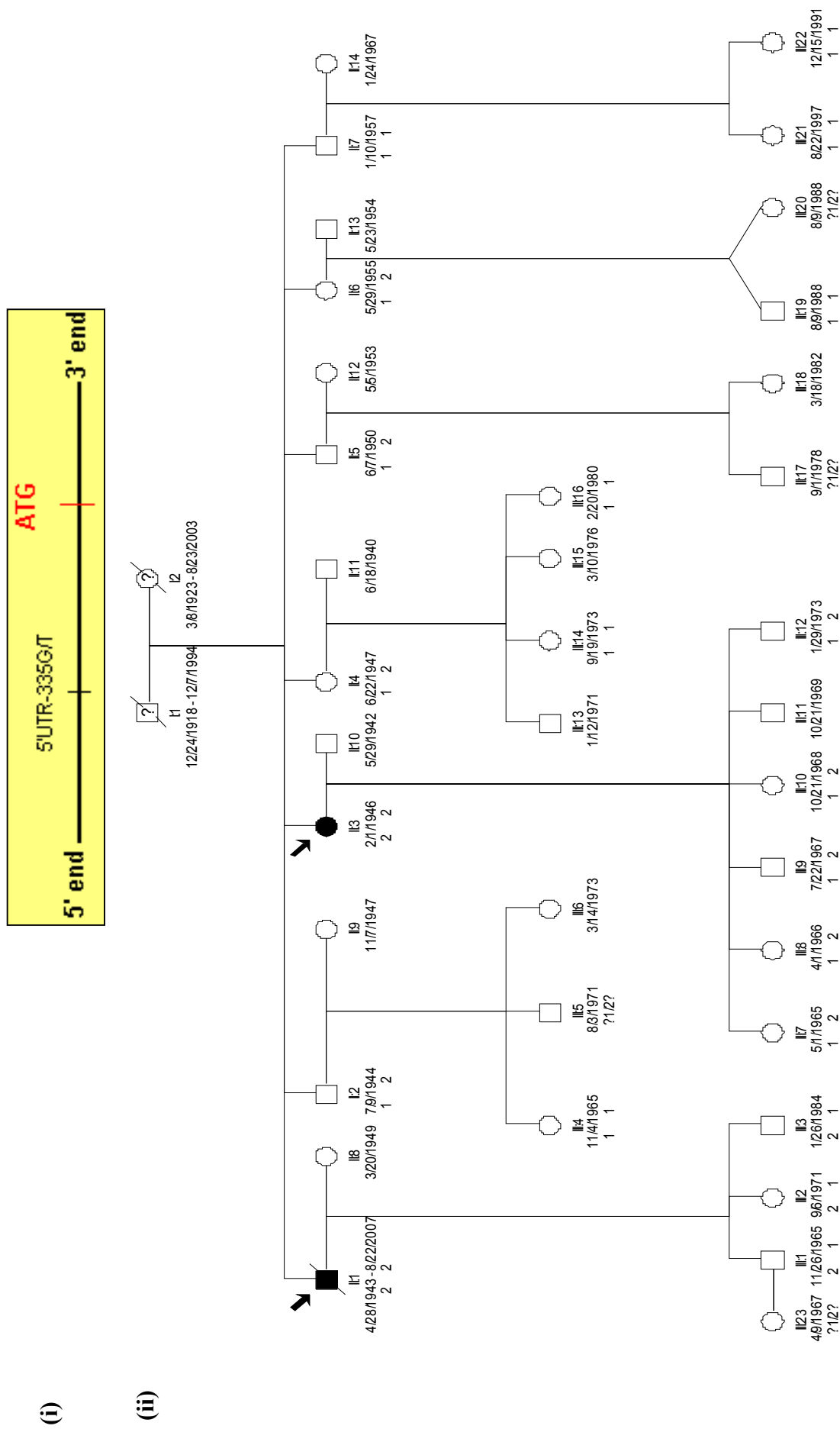
Schematic representation of haplotypes constructed for the *HFE* gene variants. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the positions of the *HFE* variants. ii) Pedigree with constructed haplotypes provided for each individual. Haplotypes are constructed in the order that the gene is annotated. Blue, haplotype 1; brown, haplotype 2; pink, haplotype 3. Abbreviations: 3', 3-prime end; 5', 5-prime end; ATG, translation initiation site; D, aspartic acid; H, histidine; *HFE*, high-iron gene; UTR, untranslated region.

3.2.4 *HAMP* gene

Mutation analysis of the *HAMP* gene revealed a single known variant in the 5'UTR (5'UTR-335G→T) (HapMap; refSNP ID: rs3817623). This variant is a transversion of a G to T nucleotide at position 335 upstream relative to the initiating ATG. The variant was identified following HEX-SSCP and sequencing analysis of promoter fragment 4 of the gene and was observed in the homozygous state in both of the probands. None of the extended family members were homozygous for the variant allele but the heterozygous state was observed in 16 (69.6%) of these individuals.

Although only a single variant was identified in the *HAMP* gene, this variant was used to construct haplotypes for the Indian family. Annotation of the gene and the haplotypes constructed for the *HAMP* variant are shown in Figure 3.4.

Figure 3.4. Haplotypes constructed for the *HAMP* gene



Legend to Figure 3.4.

Schematic representation of haplotypes constructed for the *HAMP* gene variant. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the position of the variant. ii) Pedigree with constructed haplotypes provided for each individual. Abbreviations: 3', 3-prime end; 5', 5-prime end; ATG, translation initiation site; *HAMP*, hepcidin antimicrobial peptide gene; UTR, untranslated region.

3.2.5 *SLC40A1* gene

Five previously identified variants were identified in the *SLC40A1* gene (5'UTR-1355G→C, 5'UTR-750G→A, 5'UTR-593C→T, 5'UTR-501T→C and V221). The CGG microsatellite within the *SLC40A1* promoter was also identified with either 7 or 8 repeats.

HEX-SSCP analysis and sequencing of fragment 1 of this gene promoter revealed a known nucleotide substitution. The G to C transversion at nucleotide position 1355 upstream from the initiating ATG (5'UTR-1355G→C) (HapMap; refSNP ID: rs3811621) was identified in the heterozygous state in both of the probands. Screening of the extended family members revealed eight (34.8%) homozygotes and 13 (56.5%) heterozygotes.

A known G to A transition at nucleotide position 750 upstream from ATG (5'UTR-750G→A) (HapMap; refSNP ID: rs13015236) was identified in the homozygous state in proband 1 and in the heterozygous state in proband 2. Subsequent screening of the extended family members revealed 14 (60.9%) heterozygous and five (21.7%) homozygous individuals.

Direct sequencing analysis of the 840 bp *SLC40A1* fragment revealed a nucleotide substitution of a C to a T at position 593 upstream from the initiating ATG. This variant was identified in the homozygous variant state in both of the probands. The variant (5'UTR-593C→T) (HapMap; refSNP ID: rs12693542) presented in the heterozygous form in nine (39.1%) family members, while the homozygous variant genotype was observed in 13 (56.5%) individuals.

Sequencing analysis of 840 bp fragment revealed another variant. The 5'UTR-501T→C variant (HapMap; refSNP ID: rs6728200) is a T to C transition 501 nucleotides upstream from the initiating ATG. Both of the probands as well as all of the family members genotyped were homozygous for the variant C allele.

HEX-SSCP and sequencing analysis of the coding region of the *SLC40A1* gene revealed a single synonymous variant in exon 6. The variant is a T to C transition at nucleotide position 663 (g.663 T→C) that does not result in the replacement of the amino acid valine at amino acid position 221 (V221) (Devalia *et al*, 2002). This variant was identified in the heterozygous state in proband 1 but was absent from proband 2. Genotyping of the extended family members revealed 16 (69.6%) heterozygous individuals but the homozygous variant state was not observed.

The previously described CGG microsatellite in the 5'UTR (Lee *et al*, 2001) was identified after direct sequencing of the 840 bp fragment. The repeat was observed in two allelic forms namely, allele 1: (CGG)₈ and allele 2: (CGG)₇. The two probands were both homozygous for (CGG)₈ (allele 1). Eleven (47.8%) of the family members were also homozygous for allele 1. Genotyping further revealed 12 (52.2%) individuals who were heterozygous having both the (CGG)₇ and (CGG)₈ alleles. The homozygous (CGG)₇ genotype was not observed.

The *SLC40A1* gene annotation and the haplotypes constructed for the variants identified within this gene are shown in Figure 3.5.

Legend to Figure 3.5.

Schematic representation of haplotypes constructed for the *SLC40A1* gene variants. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the positions of the variants. ii) Pedigree with constructed haplotypes provided for each individual. Haplotypes are constructed in the order that the gene is annotated. Blue, haplotype 1; pink, haplotype 2; purple, haplotype 3. Abbreviations: 3', 3-prime end; 5', 5-prime end; ATG, translation initiation site; *SLC40A1*, solute carrier family 40 (iron-regulated transporter) member 1 gene; UTR, untranslated region; V, Valine.

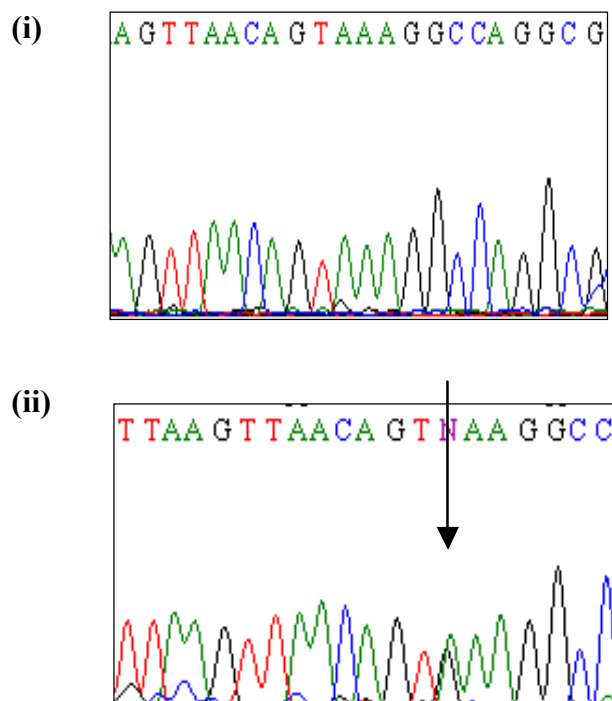
3.2.6 *CYBRD1* gene

Mutation analysis of the *CYBRD1* promoter and coding region revealed ten previously described (5'UTR-1844C→G, 5'UTR-1834G→A, 5'UTR-1813C→T, 5'UTR-1459T→C, 5'UTR-624G→A, 5'UTR-238A→G, 5'UTR-167C→G, 5'UTR-163G→A, IVS2+8T→C and S266N) and two novel (5'UTR-1452T→C and 5'UTR-1272T→C) variants. A repeat was also observed in the 5'UTR [G(T)₈G(T)₆G(T)_nG(T)₉] where n represents either zero or six repeats.

Direct sequencing analysis of the 747 bp *CYBRD1* promoter fragment, amplified using BP1 forward and BP3 reverse primers according to Table 2.5, revealed four previously described variants. The first was a single nucleotide substitution observed 1844 nucleotides upstream from the initiating codon (5'UTR-1844C→G) (HapMap; refSNP ID: rs7585974). This variant was observed in both of the probands in the homozygous state. Subsequent genotyping of the extended family revealed 17 (73.9%) homozygous and five (21.7%) heterozygous individuals. The homozygous wild type genotype was not observed in this Indian family. The second known variant 5'UTR-1834G→A (HapMap; refSNP ID: rs7586174) was observed 1834 nucleotides upstream from the initiating ATG. This variant was identified in the heterozygous state in both of the probands. Four (17.4%) family members were homozygous for this variant and 12 (52.2%) were heterozygous. The third previously described variant was a C to T transition 1813 nucleotides upstream from the first ATG codon (5'UTR-1813C→T) (HapMap; refSNP ID 12692965). This variant was absent from the two probands but was observed in the heterozygous state in five (21.7%) family members. This variant was not detected in the homozygous form. The fourth known single nucleotide substitution (T to C) was identified 1459 nucleotides upstream relative to the ATG (5'UTR-1459T→C). This previously described variant (HapMap; refSNP ID: rs10199858) was observed in the heterozygous state in both of the probands. Genotyping of the extended family revealed 10 (43.5%) heterozygous and four (17.4%) homozygous individuals.

A second novel variant was identified following HEX-SSCP analysis of fragment 4 of the *CYBRDI* promoter. A T to C transition was observed at nucleotide position 1272 upstream of the initiating ATG (5'UTR-1272T→C). Both probands were heterozygous for this variant. The variant was also found in the heterozygous state in five (21.7%) extended family members. The homozygous genotype was not identified in the family. The HEX-SSCP gel showing the heteroduplexes for the variant and the sequencing electropherogram of this variant are shown in Figure 3.8.

Figure 3.7. Schematic representation of the novel 5'UTR-1452T→C variant in the *CYBRDI* promoter



Legend to Figure 3.7.

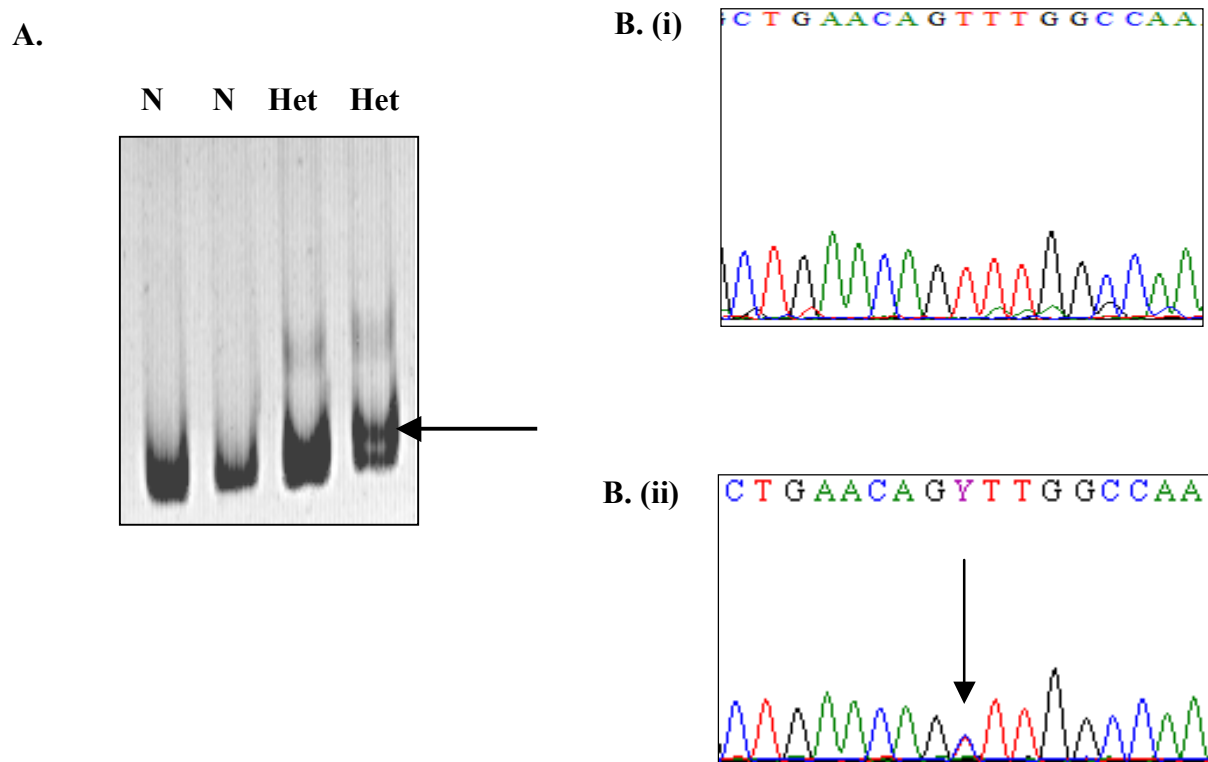
Sequencing electropherograms indicating (i) the wild type sequence and (ii) the 5'UTR-1452T→C variant. Arrow indicates the point of variation, *green* adenine or A, *blue* cytosine or C, *black* guanine or G, *red* thymine or T.

A G to A transition 624 nucleotides upstream from the initiating ATG codon (5'UTR-624G→A) (HapMap; refSNP ID: rs884408) was identified in the heterozygous state in both of the probands. Family member genotyping of this previously described variant revealed one (4.3%) homozygous and 12 (52.2%) heterozygous individuals.

A known variant, which is an A to T transition, was identified 238 nucleotides upstream from the initiating ATG (5'UTR-238A→G) (HapMap; refSNP ID: rs868106). This variant was

observed in the heterozygous state in both of the probands as well as in 12 (52.2%) family members, furthermore, the variant presented in the homozygous state in three family members (13%).

Figure 3.8. Schematic representation of the novel 5'UTR-1272T→C variant in the *CYBRD1* promoter



Legend to Figure 3.8.

A. HEX-SSCP gel stained with EtBr and visualised using ultraviolet light transillumination. Arrow indicates aberrant band observed in the heteroduplex upon HEX-SSCP analysis, Het = heterozygous banding pattern, N = homozygous wild type banding pattern. **B.** Sequencing electropherograms indicating (i) the wild type sequence and (ii) the 5'UTR-1272T→C variant. Arrow indicates the point of variation, *green* adenine or A, *blue* cytosine or C, *black* guanine or G, *red* thymine or T.

Following HEX-SSCP and sequencing analysis of fragment 10 of the promoter two previously described variants were identified. The first was a C to G transversion at nucleotide position 167 upstream from the initiating ATG (5'UTR-167C→G) (HapMap; refSNP ID: rs2356782). This variant was observed in the heterozygous state in both probands as well as in 10 family members. Four family members (17.4%) presented with the homozygous state of this variant. The second variant was a G to A nucleotide substitution 163 nucleotides upstream relative to ATG (5'UTR-163G→A) (HapMap; refSNP ID: rs3731976).

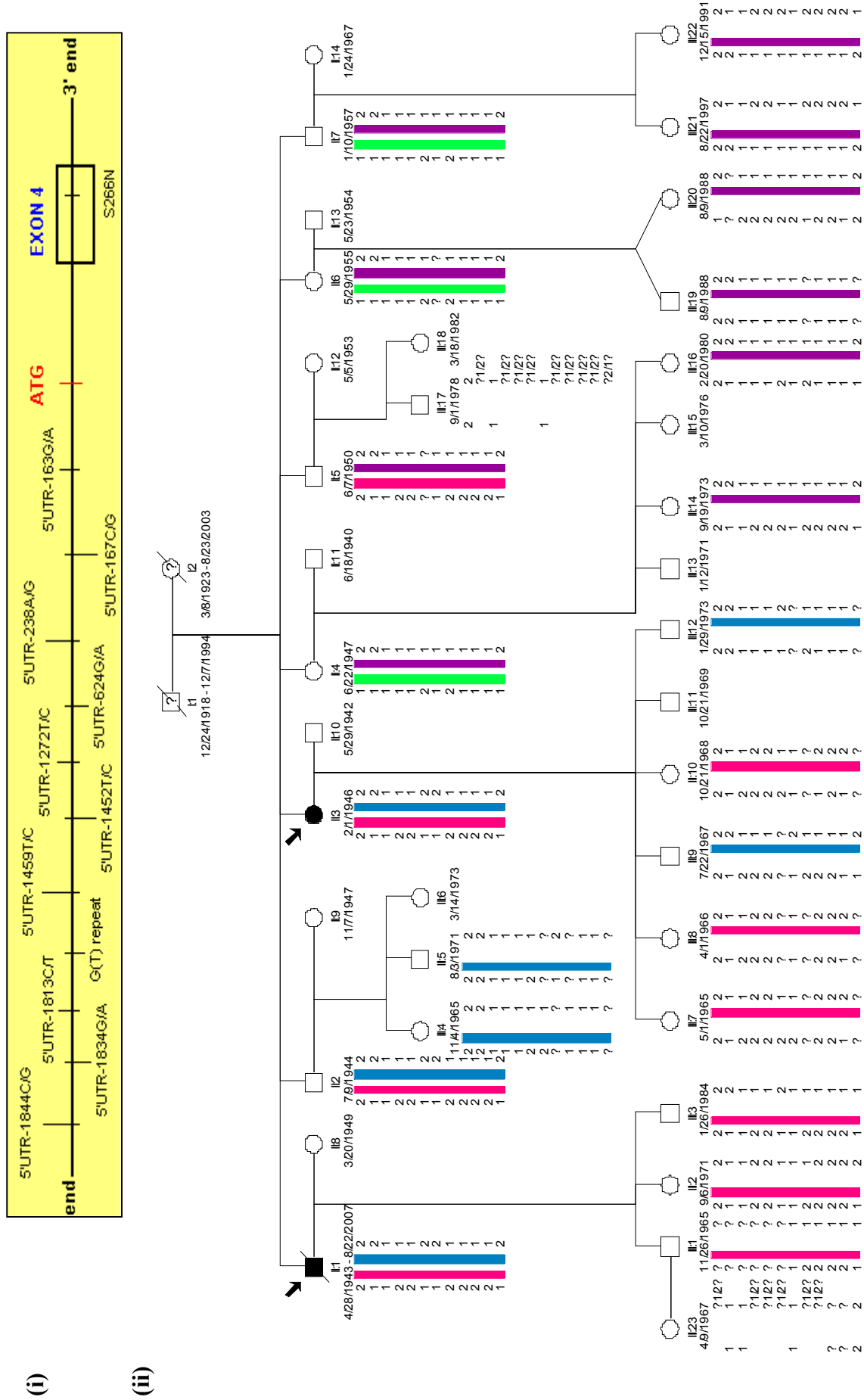
Both probands were heterozygous for this variant. One family member (4.3%) was homozygous for this variant while 11 (47.8%) were heterozygous.

Mutation analysis of exon 2 of the *CYBRDI* gene revealed a previously described intronic variant (IVS2+8T→C), located in intron 2 (Zaahl *et al*, 2004). This non-coding variant was identified in the heterozygous state in both of the probands. Due to inconclusive results following HEX-SSCP mutation analysis, genotyping of this variant in the extended family members was incomplete. This variant was omitted from haplotype analysis due to the inconclusive genotyping results. The extended family members should be genotyped for this variant employing semi-automated DNA sequencing analysis but due to financial constraints, this was not done in the present study.

A previously described exonic variant, which replaces serine with asparagine at amino acid position 266 (S266N) (McKie *et al*, 2001), was identified in exon 4 of the *CYBRDI* gene. This variant results from a G to A transition at nucleotide position 797 (g.797G→A) and was observed in the heterozygous state in both probands. This variant abolishes a *TspRI* recognition site (5' CAG↓TG 3'); therefore the extended family members were subjected to RFLP analysis for genotyping of this variant. The homozygous wild type genotype produced DNA fragments of 130 bp and 87 bp. The heterozygous genotype produced three fragments of 217 bp, 130 bp and 87 bp, respectively while the homozygous variant genotype only produced a single DNA fragment of 217 bp. Twelve family members (52.2%) were heterozygous for this variant and two (8.7%) were homozygous for the variant allele.

The gene annotation and haplotypes constructed for 12 of the 13 *CYBRDI* variants are depicted in Figure 3.9.

Figure 3.9. Haplotypes constructed for *CYBRD1* gene



Legend to Figure 3.9.

Schematic representation of haplotypes constructed for the *CYBRDI* gene variants. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the positions of the *CYBRDI* variants. ii) Pedigree with constructed haplotypes provided for each individual. Haplotypes are constructed in the order that the gene is annotated. Pink, haplotype 1; blue, haplotype 2; green, haplotype 3; purple, haplotype 4. Abbreviations: 3', 3-prime end; 5-prime end; 3', ATG, translation initiation site; *CYBRDI*, cytochrome b reductase 1 gene; IVS, intervening sequence; N, asparagine, S, serine; UTR, untranslated region.

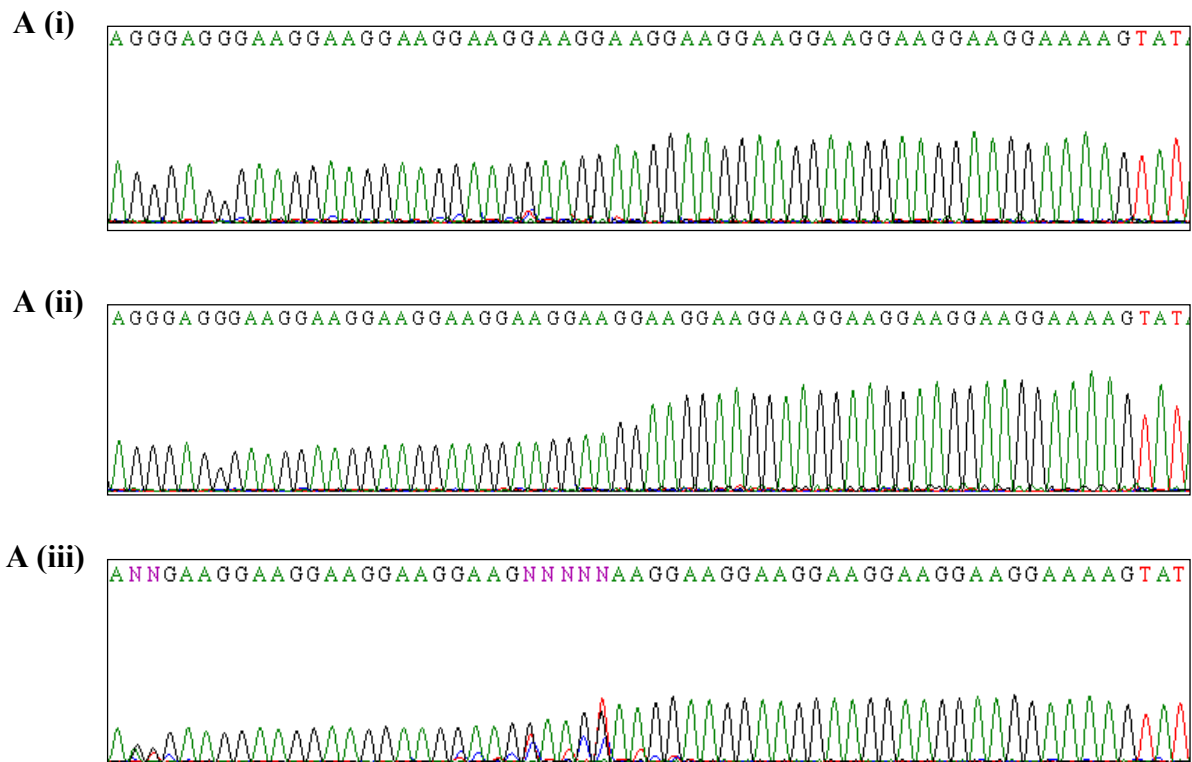
3.2.7 *HJV* gene

Mutation analysis of the *HJV* gene revealed two previously described (5'UTR-1406C→A and 5'UTR-542A→G) and two novel variants (5'UTR-534G→T and 5'UTR-530G→T) in the *HJV* gene promoter. An AAGG variable number tandem repeat (VNTR) or microsatellite was identified in the promoter region with 11 to 13 repeats. No variants were identified in the coding region of the *HJV* gene.

Following HEX-SSCP and sequencing analysis of fragment 1 of the *HJV* promoter a known C to A transversion was observed 1406 nucleotides from the initiating ATG (5'UTR-1406C→A) (HapMap; refSNP ID: rs1830823). Both the probands were heterozygous for this variant as well as 16 family members (69.6%). The variant was identified in the homozygous state in two family members (8.7%).

HEX-SSCP analysis of promoter fragment 6 (Table 2.6) did not yield clear results for genotyping so direct sequencing analysis was performed on this fragment for the two probands and extended family members. Sequencing analysis of this fragment revealed three variants in the 5'UTR. A previously described variant (5'UTR-542A→G) (HapMap; refSNP ID: rs10910811) is an A to G transition at nucleotide position 542 upstream relative to the initiating ATG. This variant was observed in the heterozygous form in both of the probands. Genotyping of the extended family members revealed nine (39.1%) heterozygotes and five (21.7%) homozygotes. Two novel G to T transversions were identified 534 and 530 nucleotides upstream from ATG, respectively (5'UTR-534G→T and 5'UTR-530G→T). The 5'UTR-534G→T variant was observed in the heterozygous state in both of the probands and in 14 (60.9%) family members. Both probands were also heterozygous for the 5'UTR-530G→T variant as well as 14 (60.9%) of the extended family members. The homozygous state of both of these variants was not identified. The schematic representation of the 5'UTR-534G→T and 5'UTR-530G→T variants are shown in Figure 3.10. An AAGG repeat was also

Figure 3.11. Schematic representation of the novel AAGG repeat identified in the *HJV* promoter



Legend to Figure 3.11.

A. Sequencing electropherograms indicating (i) allele 1 (AAGG)₁₁ (ii) allele 2 (AAGG)₁₂ and (iii) allele 3 (AAGG)₁₃ (in the heterozygous state with allele 2) of the repeat in the *HJV* promoter. *Green* adenine or A, *blue* cytosine or C, *black* guanine or G, *red* thymine or T.

The gene annotation for *HJV* and the haplotypes assembled for the *HJV* single nucleotide substitutions and repeat are shown in Figure 3.12.

Legend to Figure 3.12.

Schematic representation of haplotypes constructed for the *HJV* gene variants. i) Gene annotation indicating the transcription initiation site and the position of variants used as markers for haplotype assembly. Gene annotation is not drawn to scale and only indicates the positions of the *HJV* variants. ii) Pedigree with constructed haplotypes provided for each individual. Haplotypes are constructed in the order that the gene is annotated. Blue, haplotype 1; pink, haplotype 2; purple, haplotype 3; green, haplotype 4. Abbreviations: 3', 3-prime end; 5', 5-prime end; ATG, translation initiation site; *HJV*, hemojuvelin gene; UTR, untranslated region.

3.3 Summary of Haplotype Analysis

The construction of haplotypes gives an indication of which haplotypes could be modifying the iron overload phenotype. Haplotypes that were identified in the two probands as well as unaffected family members suggest that this haplotype is not associated with disease development and can be excluded. Those haplotypes that were unique to the two probands were included and could be associated with the iron overload phenotype. Table 3.3 is a summary of the results obtained following haplotype analysis.

Table 3.3. Summation of haplotype analysis

Gene	Haplotype similarity in Proband 1 and Proband 2	Family members with same haplotype as probands		Include (√) or exclude (×) haplotype*
		Proband 1	Proband 2	
<i>HMOX1</i>	Differ	None	None	√
<i>HFE</i>	Same	None	None	√
<i>HAMP</i>	Same	None	None	√
<i>SLC40A1</i>	Differ	II:2, II:6	II:5	×
<i>CYBRD1</i>	Same	II:2	II:2	×
<i>HJV</i>	Same	II:5, II:7	II:5, II:7	×

Abbreviations: *CYBRD1*, cytochrome b reductase 1 gene; *HAMP*, hepcidin antimicrobial peptide gene; *HFE*, high-iron gene; *HJV*, hemojuvelin gene; *HMOX1*, haem oxygenase-1 gene; *SLC40A1*, solute carrier family 40 (iron-regulated transporter) member 1 gene. *Haplotypes were excluded if they were present in the probands as well as unaffected family members. The haplotypes were not contributing to the disease phenotype when considered on their own, but could still be associated with disease when considered in conjunction with other yet unidentified modulating genes.

3.4 *In Silico* Analysis of Exonic Variants

A previously described silent variant was identified in the *SLC40A1* gene. This single nucleotide substitution preserves the valine amino acid at amino acid position 221 (V221) (Devalia *et al*, 2002). Another known exonic variant was identified in exon 4 of the *CYBRDI* gene. This nonsense mutation results in the replacement of the polar, hydrophilic amino acid serine with polar, uncharged asparagine at amino acid position 266 (S266N) (McKie *et al*, 2001). The ESEfinder (ESE-Exonic Splice Element) program was used to analyse these variants to determine their possible effect on splicing (Cartegni *et al*, 2003; Smith *et al*, 2006) (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi>). No alteration in splicing was predicted in the presence of the variant allele of either the V221 variant or the S266N variant.

3.5 *In Silico* Analysis of Intronic Variants

Three known intronic variants were identified in this study. They included two identified in intron 2 of the *HMOX1* gene (IVS2+4T→C and IVS2-19C→T) and one identified in intron 2 of the *CYBRDI* gene (IVS2+8T→C). These intronic variants were subjected to analysis using the Alternative Splice Site Predictor (ASSP) program (Wang and Marín, 2006) (<http://www.es.embnnet.org/~mwang/assp.html>) to determine if splicing was affected in the presence of the variant allele. All the donor and acceptor sites present in the wild type sequences were preserved within the variant sequences. Therefore, this program did not predict any altered splicing patterns resulting from any of these variants.

3.6 *In Silico* Promoter Analysis

Variants identified within the 5'UTR of the various genes under investigation were subjected to *in silico* analysis to determine whether they disrupted any transcription factor binding sites (TFBS). Several programs are available for *in silico* analysis of gene regulatory regions. The TRANSFAC®7 database (<http://www.gene-regulation.com/pub/databases.html#transfac>) (Wingender *et al*, 2001) contains information on many transcription factors as well as their experimentally verified binding sites. All promoter variants were analysed using the PATCH program (<http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi>), available within the TRANSFAC®7 database to identify putative TFBS that may be disrupted by these variants. The 5'UTR variants were also analysed using the non-redundant JASPAR

CORE database (<http://jaspar.genereg.net/>) (Sandelin *et al*, 2004). The default settings and parameters of these two software programs were applied when ascertaining if a nucleotide substitution disrupted a putative TFBS. Putative TFBS identified using PATCH and JASPAR CORE were analysed using the rVISTA program. The rVISTA program (<http://genome.lbl.gov/vista/rvista/submit.shtml>) (Loots *et al*, 2002) is another program available within the TRANSFAC®7 database. This program identifies conserved binding motifs and was employed to verify results obtained using PATCH and JASPAR CORE. The results obtained through *in silico* analysis of the promoter variants identified in each of the genes are shown in Tables 3.4-3.9.

Table 3.4. Results from *in silico* data analysis of 5'UTR *HMOX1* variant

Variant	Putative Transcription Factor Binding Sites				rVISTA Results		
	PATCH		JASPAR		Conserved Sites	Sites Abolished in presence of Variant	Sites Created in Presence of Variant
	Abolished	Created	Abolished	Created			
5'UTR-495A→T	-	-	FOXL1, SOX9	HNF4A	FOXL1, SOX9, HNF4A	-	HNF4A

Abbreviations: 5', 5-prime end; FOXL1, forkhead box protein L1; *HMOX1*, haem oxygenase-1 gene; HNF4A, hepatocyte nuclear factor 4- α ; SOX9, Sry-related high-mobility group (HMG) box-9; UTR, untranslated region.

Table 3.5. Results from *in silico* data analysis of 5'UTR *HFE* variants

Variant	Putative Transcription Factor Binding Sites						rVISTA Results		
	PATCH			JASPAR			Conserved Sites	Abolished Sites	Created Sites
	Abolished	Created	Created	Abolished	Created	Created			
5'UTR-1206C→G	RXR- α , VDR, RAR- α 1, RAR- β ,	CTCF		-	-	-	VDR	-	VDR
5'UTR-840T→G	-	SPI		PU.1	-	-	SPI, PU.1	-	-
5'UTR-467G→C	PPUR	-	-	-	MAX, USFI	-	USF	-	USF

Abbreviations: 5', 5-prime end; CTCF, CCCTC binding factor; *HFE*, high-iron gene; IKZF1, ikaros family zinc finger 1; MAX, Myc-associated factor X; PU.1, spleen focus forming virus proviral integrating oncogene 1; RAR- α 1, retinoic acid receptor α 1; RAR- β , retinoic acid receptor β ; RXR- α , retinoid X receptor α ; SPI, specificity protein 1; USF1, upstream stimulatory factor 1; UTR, untranslated region; VDR, vitamin D3 receptor.

Table 3.6. Results from *in silico* data analysis of 5'UTR *HAMP* variant

Variant	Putative Transcription Factor Binding Sites				rVISTA Results		
	PATCH		JASPAR		Conserved Sites	Abolished Sites	Created Sites
	Abolished	Created	Abolished	Created			
5'UTR-335G→T	IL4, VDR	-	PU.1	-	IL4, VDR, PU.1	-	-

Abbreviations: 5', 5-prime end; *HAMP*, hepcidin antimicrobial peptide gene; IL4, interleukin-4; PU.1, spleen focus forming virus proviral integrating oncogene 1; UTR, untranslated region; VDR, vitamin D3 receptor.

Table 3.7. Results from *in silico* data analysis of 5'UTR *SLC40A1* variants

Variant	Putative Transcription Factor Binding Sites				rVISTA Results		
	PATCH		JASPAR		Conserved Sites	Abolished Sites	Created Sites
	Abolished	Created	Abolished	Created			
5'UTR-1355G→C	-	-	-	-	-	-	-
5'UTR-750G→A	STAT1, STAT3, RAR- α 1, RAR- β , RXR- α , PXR-1, RXR- β , SXR, c-myb	-	PU.1, ETS	-	STAT1, STAT3, c-myb, PU.1, ETS	-	STAT3
5'UTR-593C→T	-	-	-	CREB1	NF-AT, CREB1	-	-
5'UTR-501T→C	CACCC binding factor, SP1	SMAD3, SMAD4, SP1-4, AP2	-	AP2	SP1, SMAD3, SMAD4, SP3, AP2	-	SMAD4

Abbreviations: 5', 5-prime end; AP2, activator protein 2; c-myb, Myb proto-oncogene protein; CREB1, cAMP response element binding protein; ETS, ETS oncogene; PU.1, spleen focus forming virus proviral integrating oncogene 1; PXR-1, pregnane X receptor; RAR- α 1, retinoic acid receptor α 1; RAR- β , retinoic acid receptor β ; RXR- α , retinoid X receptor α ; RXR- β , retinoid X receptor β ; *SLC40A1*, solute-carrier family 40 (iron-regulated transporter) member 1 gene; SMAD3, mothers against decapentaplegic homolog 3; SMAD 4, mothers against decapentaplegic homolog 4; SP1-4, specificity proteins 1-4; STAT1, signal transducer and activator of transcription 1- α / β ; STAT3, signal transducer and activator of transcription 3; SXR, steroid and xenobiotic receptor; UTR, untranslated region.

Table 3.8. Results from *in silico* data analysis of 5'UTR *CYBRDI* variants

Variant	Putative Transcription Factor Binding Sites			rVISTA Results		
	PATCH		JASPAR	Conserved Sites	Abolished Sites	Created Sites
	Abolished	Created	Abolished			
5'UTR-1844C→G	-	-	FOXCl, ETS	ETS	-	-
5'UTR-1834G→A	API	VDR, RAR- α 1, RAR- β , RXR- α , PXR-1, RXR- β , SXR	-	API, VDR, PU.1	-	-
5'UTR-1813C→T	SP1, CAC binding protein, VDR	T3R, RAR- α 1, RXR- β , RAR- β , RAR- γ , RXR- α	-	SP1, T3R, VDR, PU.1	SP1, VDR	-
5'UTR-1459T→C	NF-1, AP- 2 α A, AP- 2 α B	SP1-4	HNF4A	NF-1, AP-2 α A, SP1, SP3, HNF4A	-	SP1, HNF4A
5'UTR-1452T→C	-	-	HNF4A	HNF4A	-	-
5'UTR-1272T→C	-	API, C/EBP	-	API, C/EBP	C/EBP	-
5'UTR-624G→A	-	-	AML1	AML1	-	-
5'UTR-238A→G	-	-	NF-Y	NF-Y, SP1	-	-
5'UTR-167C→G	C/EBP, API, SP1-4	ETS1, LEF1	-	API, SP1, SP3, ETS1, LEF1, ELK1	-	-
5'UTR-163G→A	SP1	API, C/EBP	-	SP1, API	-	-

Abbreviations: 5', 5-prime end; AML1, acute myeloid leukemia 1 protein; API, activator protein 1; AP-2 α A, activating enhancer-binding protein 2- α ; AP-2 α B, activating enhancer-binding protein 2- β ; C/EBP, CCAAT/enhancer binding protein; *CYBRDI*, cytochrome b reductase 1 gene; ELK1, ETS domain-containing protein ELK1; ETS, ETS oncogene; ETS1, protein C-ets-1; FOXCl, forkhead box protein C1; GABP α , GA-binding protein α chain; HNF4A, hepatocyte nuclear factor 4- α ; LEF1, lymphoid enhancer-binding factor 1; NF-1, neurofibromin; NF-Y, nuclear factor Y; PU.1, spleen focus forming virus proviral integrating oncogene 1; PXR-1, pregnane X receptor; RAR- α 1, retinoic acid receptor α 1; RAR- β , retinoic acid receptor β ; RAR- γ , retinoic acid receptor γ ; RUNX2, runt-related transcription factor 2; RXR- α , retinoid X receptor α ; RXR- β , retinoid X receptor β ; SP1-4, specificity proteins 1-4; SXR, steroid and xenobiotic receptor; T3R, thyroid hormone receptor; UTR, untranslated region; VDR, vitamin D3 receptor.

Table 3.9. Results from *in silico* data analysis of 5'UTR *HJV* variants

Variant	Putative Transcription Factor Binding Sites				rVISTA Results		
	PATCH		JASPAR		Conserved Sites	Abolished Sites	Created Sites
	Abolished	Created	Abolished	Created			
5'UTR-1406C→A	AP2, AP-2αA, AP-2αB, NF1	-	NR3C1	GATA2	AP2, AP-2αA, GATA2, NF1	AP2, NF1	-
5'UTR-542A→G	ETS2, PEA3	T3R, RXR-α, VDR	ETS, PU.1	-	ETS2, PEA3, T3R, VDR, ETS, PU.1	ETS	T3R
5'UTR-534G→T	ETS2	T3R	PU.1, ELK1	ETS, FOXCI	ETS2, T3R, PU.1, ELK1, ETS	T3R, PU.1, ELK1, ETS	-
5'UTR-530G→T		-	PU.1	-	PU.1	PU.1	-

Abbreviations: 5', 5-prime end; AP2, activator protein 2; AP-2αA, activating enhancer-binding protein 2-α; AP-2αB, activating enhancer-binding protein 2-β; ELK1, ETS domain-containing protein ELK1; ETS, ETS oncogene; ETS2, protein C-ets-2; FOXCI, forkhead box protein C1; GATA2, GATA-binding protein 2; *HJV*, hemojuvelin gene; PEA3, ETS domain-containing transcription factor PEA3; PU.1, spleen focus forming virus proviral integrating oncogene 1; RXR-α, retinoid X receptor α; T3R, thyroid hormone receptor; UTR, untranslated region; VDR, vitamin D3 receptor; NF-1, neurofibromin; NR3C1, nuclear receptor subfamily 3 group C member 1.

Discussion

A number of variants were observed in the six genes investigated in this Indian family. These included 24 previously identified variants, one known repeat, five novel single nucleotide substitutions and two novel repeats. These variants were either identified in the exonic, intronic or 5'UTR of the different genes under investigation. The 24 previously identified and the five novel variants all resulted from single nucleotide substitutions.

Single nucleotide polymorphisms (SNPs) are single nucleotide positions within genomic DNA at which two or more different alleles occur in the general population. The least frequent allele must be present in 1% or more of a specific population for a nucleotide variation to be classified as a SNP. SNPs occur at a frequency of approximately one per 1000 bp in the human genome (Taillon-Miller *et al*, 1998) and account for nearly 90% of all genetic variation observed (Collins *et al*, 1998). SNPs in the coding regions of genes or in their regulatory regions are more likely to be functional than those found in other regions. Association studies focussed on identifying candidate genes that may be involved in disease development are expensive as they involve searching the entire genome for these disease associations. SNPs are invaluable markers that can be employed to reduce the search region for disease causing loci from the whole genome to a smaller region associated with the disease. Finding SNPs in different populations, which could be associated with genes conferring susceptibility to disease in these populations, is thus essential.

The HapMap project (The International HapMap Consortium, 2003) (<http://www.hapmap.org>) has developed a publicly accessible map of DNA sequence variation within the human genome and is still adding SNPs to the database. The study includes 270 individuals from three populations namely, European, Asian and African. Subjects include 30 family trios (two parents and an adult child) from Nigeria (African population), 45 unrelated Japanese and 45 unrelated Han Chinese individuals (Asian populations) and 30 family trios represented by the *Centre d'Etude du Polymorphisme Humaine* (CEPH) samples (European population). The CEPH samples came from a Utah population and individuals were of either northern or western European descent. This project aims to determine the frequency of SNPs in these populations and the degree of association between them. SNPs may occur with varying frequencies in different populations. Although

not all populations are represented, this project is an essential tool for the discovery of genetic variation and is a starting point for follow-up studies, in other populations, where this variation may be associated with health and disease.

As mentioned previously, HH appears to be rare in the Indian population and very few studies have investigated the genetic factors involved in the pathogenesis of the disease. As the present study included the analysis of only a single Indian family, results obtained for the respective variants are not an indication of their frequencies in the general Indian population. The International HapMap Consortium does not include SNP information for the Asian Indian population and therefore results obtained in the present study were not compared to results included in the HapMap project. Allele frequencies of known variants obtained in the HapMap project are however listed in Table 3.10 to give an indication of the prevalence of these variants in different populations.

Table 3.10. HapMap allele frequencies for known variants identified in this study

Gene	Variant	HapMap refSNP ID	Population	HapMap Allele Frequencies	
				Wild Type Allele*	Variant Allele*
<i>HMOX1</i>	5'UTR-495A→T	rs2071746	European	0.609-0.542	0.391-0.458
			African-American	0.174-0.300	0.700-0.826
			Asian	0.523-0.474	0.477-0.526
			Sub-Saharan African	0.308	0.692
	IVS2+4T→C	rs17885925	European	0.978	0.022
			African-American	0.935	0.065
	IVS2-19C→T	rs7879606	European	0.977	0.023
			African-American	0.938	0.062
<i>HFE</i>	5'UTR-1206C→G	rs1800702	European	0.479-0.600	0.400-0.521
			African-American	0.8	0.2
			Asian	0.229-0.330	0.670-0.771
			Sub-Saharan African	0.842	0.158
	5'UTR-467G→C	rs2794720	European	0.397	0.603
			Asian	0.670-0.733	0.267-0.330
			Sub-Saharan African	0.147	0.853
<i>HAMP</i>	5'UTR-335G→T	rs3817623	European	1.000	0
			Asian	0.956-0.966	0.034-0.044
			Sub-Saharan African	1.000	0

Table 3.10. HapMap allele frequencies for known variants identified in this study (Cont.)

Gene	Variant	HapMap refSNP ID	Population	HapMap Allele Frequencies	
				Wild Type allele*	Variant allele*
<i>SLC40A1</i>	5'UTR-1355G→C	rs3811621	European	0.725-0.729	0.271-0.275
			African-American	0.478	0.522
			Asian	0.838-0.896	0.104-0.162
			Sub-Saharan African	0.608	0.392
	5'UTR-750G→A	rs13015236	European	0.400	0.600
			African American	0.770	0.230
			Asian	0.270-0.310	0.690-0.730
	5'UTR-593C→T	rs12693542	European	0.293	0.707
			Asian	0.102-0.122	0.878-0.898
			Sub-Saharan African	0.551	0.449
	5'UTR-501T→C	rs6728200	Not Provided	Not Provided	Not Provided
<i>CYBRDI</i>	5'UTR-1844C→G	rs7585974*	Not Provided	Not Provided	Not Provided
	5'UTR-1834G→A	rs7586174	Not Provided	Not Provided	Not Provided
	5'UTR-1813C→T	rs12692965	Not Provided	Not Provided	Not Provided
	5'UTR-1459T→C	rs10199858	Not Provided	Not Provided	Not Provided
	5'UTR-624G→A	rs884408	Not Provided	Not Provided	Not Provided
	5'UTR-238A→G	rs868106*	European	0.370-0.500	0.500-0.630
			African-American	0.310	0.690
Asian			0.045-0.103	0.897-0.955	
5'UTR-167C→G	rs2356782	European	0.440	0.560	
		Asian	0.160	0.840	
5'UTR-163G→A	rs3731976	European	0.630	0.370	
		Asian	0.280	0.720	
IVS2+8T→C	rs7586144	European	0.208-0.271	0.792-0.729	
		African-American	0.275	0.725	
		Asian	0.021-0.176	0.979-0.824	
		Sub-Saharan African	0.158-0.167	0.842-0.833	
S266N (g.797G→A)	rs10455	European	0.258-0.271	0.729-0.742	
		African-American	0.109	0.891	
		Asian	0.580-0.708	0.292-0.420	
		Sub-Saharan African	0.042	0.958	
<i>HJV</i>	5'UTR-1406C→A	rs1830823	Not Provided	Not Provided	Not Provided
	5'UTR-542A→G	rs10910811	Not Provided	Not Provided	Not Provided

Abbreviations: 5'UTR, 5-prime untranslated region; *CYBRDI*, cytochrome b reductase 1 gene; *HAMP*, hepcidin antimicrobial peptide gene; *HFE*, high-iron gene; *HJV*, hemojuvelin gene; *HMOX1*, haem oxygenase-1 gene; IVS, intervening sequence; N, asparagine; S, serine; *SLC40A1*, solute-carrier family 40 (iron-regulated transporter) member 1 gene. *The wild type and variant alleles are as indicated in the naming of each variant (wild type allele → variant allele).

Many variants were identified after mutation analysis of this Indian family. All variants identified in a single gene were considered together to construct haplotypes using the Cyrillic 2.01 program (Cherwell Scientific Publishing Ltd, Magdalen Centre, Oxford Science Park, Oxford, UK). Haplotypes give a clearer indication of which variants identified in a gene may be functioning in concert and subsequently causing iron overload or modulating the HH

phenotype. The results obtained after haplotype analysis of each gene will be discussed further.

***HMOX1* gene**

Three previously described variants were identified after mutation analysis of the *HMOX1* promoter and coding regions. These included one promoter variant, 5'UTR-495A→T (HapMap; refSNP ID: rs2071746) and two intronic variants, IVS2+4T→C (HapMap; refSNP ID: rs 17885925) and IVS2-19C→T (HapMap; refSNP ID: rs 7879606) identified in intron 2 of the gene.

Probands 1 and 2 presented with two different haplotypes: proband 1 was heterozygous for the 5'UTR-495A→T and IVS2+4T→C variants and homozygous wild type for the IVS2-19C→T variant while proband 2 was heterozygous for the promoter variant as well as both of the intronic *HMOX1* variants (refer to Figure 3.2). Neither of these haplotypes was present in any of the extended family members.

Both probands developed HH symptoms in the fifth decade of life. Individuals II:4, II:5, II:6 and II:7 are all older than 50 years, heterozygous for the promoter variant, homozygous wild type for both intronic variants and did not present with elevated iron levels. These data indicate that heterozygosity for the 5'UTR-495A→T alone does not seem to be associated with disease development. Third generation family members III:8 and III:10 (see Figure 3.2) were heterozygous for both intronic variants as well as the promoter variant. Individuals III:8 and III:10 are both females with ages of 41 and 39 years, respectively. Individual III:8 presented with a TS% of 26% and a SF level of 118 µg/l. Individual III:10 presented with a TS% of 33% and a SF level of 213 µg/l. Because of the young age of these individuals, the iron overload phenotype may not be fully expressed. Individual III:10 does have a slightly elevated SF level (female reference range: 20-200 µg/l) but individual III:8 (same haplotype as III:10) does not seem to be displaying signs of iron overload and this presumably indicates that this haplotype may possibly not be associated with iron overload. The two probands presented with the variant allele for the promoter variant and were both heterozygous for the IVS2+4T→C variant. The IVS2-19C→T variant was not present in proband 1 and therefore does not seem to be associated with the HH phenotype and could be a common polymorphism in this family. Inheritance of the 5'UTR-495A→T variant allele (heterozygous state) and the

IVS2+4T→C variant could be modifying the iron overload phenotype observed in both of the probands but this needs to be investigated further.

In silico analysis of the IVS2+4T→C intronic variant was performed. The ASSP program (Wang and Marín, 2006) (<http://www.es.embnet.org/~mwang/assp.html>) was employed to assess whether the intronic variant altered splicing of the *HMOX1* gene. All splice donor and acceptor sites remained in tact in the presence of the variant allele indicating that this variant does not affect the splicing mechanism. Functional analysis of this variant using the minigene assay for intronic variants described by Baralle *et al* (2003) is necessary to determine if this variant is contributing to the disease phenotype.

In silico analysis of the 5'UTR-495A→T variant using the JASPAR CORE program (<http://jaspar.genereg.net/>) (Sandelin *et al*, 2004) revealed that in the presence of the variant allele the putative TFBS for the Sry-related high-mobility group (HMG) box-9 (SOX9) and forkhead box protein L1 (FOXL1) transcription factors are both abolished. The program also predicted the creation of a putative hepatocyte nuclear factor 4- α (HNF4A) TFBS. To determine whether any of these putative TFBS are conserved within the human *HMOX1* gene, *in silico* analysis was performed using the rVISTA program (<http://genome.lbl.gov/vista/rvista/submit.shtml>) (Loots *et al*, 2002). The three TFBS were conserved within the *HMOX1* promoter but this program only confirmed the creation of the putative HNF4A transcription factor (See Table 3.4).

SOX9 is a member of the SOX family of transcription factors. Members of this family all exhibit the same DNA-binding domain, which is known as the high-mobility group (HMG) box. SOX transcription factors bind to the minor groove of DNA and bend and unwind the DNA. This DNA bending seems to be essential for DNA transcription to occur (reviewed by Marshall and Harley, 2000). SOX9 plays an important role in human sex determination and chondrogenesis. Mutations in the *SOX9* gene are responsible for the development of Campomelic dysplasia. This condition results in XY individuals being either intersex or developing male-to-female sex reversal. The SOX9 transcription factor has not been shown to regulate *HMOX1* gene expression or be associated with iron overload and therefore loss of this putative TFBS does not seem to be a contributing factor to developing iron overload and therefore HH.

The FOXL1 transcription factor is a member of the winged helix/forkhead family of DNA binding factors and is only expressed in the gastrointestinal mesenchyme. This transcription factor is necessary for the maintenance of the gut (Kaestner *et al*, 1996). FOXL1 has been shown to regulate the proliferation and differentiation of the gut epithelium. FOXL1 seems to regulate the expression of factors that mediate epithelial-mesenchymal interactions and defects in the epithelium result when this precise control of expression is disrupted in the mesenchyme. Interestingly, Kaestner and colleagues (1997) observed that in Foxl1-deficient mice the expression of both bone morphogenetic protein 2 (BMP2) and BMP4 were drastically reduced. Therefore FOXL1 seems to be a regulator of BMP2 and BMP4 expression. Interaction between FOXL1 and HMOX1 may possibly be necessary for FOXL1 to perform its regulatory function and the 5'UTR-495A→T variant, which potentially disrupts their interaction, could result in a decrease in BMP2 and BMP4 expression. BMP2 and BMP4 function in the BMP signalling pathway in which HJV acts as a coreceptor and positively regulates hepcidin expression (Babitt *et al*, 2006). HJV exhibits binding specificity for BMP2 and to a lesser degree, BMP4, in the BMP signalling pathway. By disrupting the FOXL1 and HMOX1 interaction, this promoter variant may be decreasing hepcidin expression and causing iron overload. Another possibility is that the promoter variant is modifying the HH phenotype. The HH phenotype is highly variable and not all patients develop osteoporosis of any kind. Different patterns of osteoclast and osteoblast dysregulation have been associated with osteoporosis development (Byers *et al*, 1997). BMPs are essential regulators of osteoblast differentiation and disruption of this regulatory pathway might result in inappropriate osteoblast differentiation and eventually osteoporosis.

Various hepatocyte-specific genes are regulated by hepatocyte nuclear factors (HNFs). Expression of these genes is dependent on the binding of a multitude of diverse HNF transcription factors. HNF transcription factors bind to gene regulatory regions and stimulate gene transcription (reviewed by Costa *et al*, 2003). HNF4A is a member of the steroid/thyroid nuclear receptor family and is expressed mainly in the liver but also in the kidneys and intestine. It is an essential regulator of liver metabolism and development. The 5'UTR-495A→T variant creates a putative HNF4A site in the *HMOX1* promoter. Creation of this TFBS may result in increased expression of the *HMOX1* gene. It has been reported that HMOX1 plays a protective role in many cell types protecting them against damage that may be caused by oxidative stress (Yachie *et al*, 1999). An increase in *HMOX1* expression increases the production of two strong antioxidants, bilirubin and biliverdin. Although the role

HNF4A plays in the regulation of *HMOX1* expression has not as yet been described, it has been noted that HNF4A expression is also increased in the presence of oxidative stress induced by hepatitis C virus infection (Qadri *et al*, 2006). Therefore HNF4A and HMOX1 may both function in the same antioxidant defence mechanism and HNF4A could regulate *HMOX1* gene expression. Excess iron present in HH patients catalyses the conversion of hydrogen peroxide to free radicals. These free radicals cause oxidative stress in various tissues (Andrews, 1999). The creation of an extra putative HNF4A TFBS may disrupt *HMOX1* gene expression within hepatocytes resulting in imperfect functioning of the antioxidant defence mechanism. This could ultimately result in hepatocyte damage in the presence of oxidative stress resulting from excess iron. The ALT level in proband 1 and the AST level in proband 2 were both slightly elevated at the time of diagnosis. Both of these enzymes are secreted by hepatocytes and elevated levels are observed when the liver is damaged. When proband 1 was initially diagnosed with HH he presented with established liver cirrhosis. Proband 2 has completed her phlebotomy course and her iron levels have stabilised and she has not developed liver cirrhosis. Therefore, the *HMOX1* promoter variant may not be causing iron overload but could contribute to the liver damage that results in the presence of excess iron.

***HFE* gene**

Three previously described variants (5'UTR-1206C→G, 5'UTR-467G→C and H63D) and 1 novel variant (5'UTR-840T→G) were identified in the *HFE* gene in the Indian family.

Feder and colleagues (1996) identified the variant H63D in many HH patients who carried a single C282Y mutation. Although approximately 80-90% of HH cases are the result of the C282Y homozygous genotype (Feder *et al*, 1996; Jazwinska *et al*, 1996; Beutler *et al*, 1996; Carella *et al*, 1997; Mura *et al*, 1997), 75% of individuals with one C282Y mutation are also compound heterozygotes with the H63D mutation (Robson *et al*, 2004). The homozygous H63D genotype has been implicated in a much milder form of the HH phenotype. Exactly how the H63D variant disrupts HFE function has not been elucidated yet. H63D is not located in the region of the HFE peptide that binds to the transferrin receptor (TFR) but it does form a salt bridge with the TFR-binding region of the $\alpha 2$ loop. Even though this variant forms the salt bridge, the H63D protein is still able to bind to TFR. Garewal *et al* (2005) ascertained the allele frequency of the C282Y and H63D *HFE* mutations in a North Indian population with

the beta thalassemia trait. They determined that both of these mutations are rare within the Northern Indian population and that the H63D mutation is not associated with iron overload in the beta thalassemia trait (reviewed by Poddar, 2006). In the present study, this variant was not identified in the two probands but it was observed in the heterozygous state in four unaffected family members (III:1, III:2, III:19 and III:21). These findings indicate that this variant does not seem to be associated with iron overload in this family.

Haplotype analysis of the *HFE* gene revealed that only three haplotypes were inherited by the second generation family members. This indicated that the parents of the two probands presented with a common haplotype. The parents of the two probands were first cousins and therefore the presence of a shared haplotype is possible. The two probands presented with the same haplotypes. Both probands were heterozygous for the 5'UTR-1206C→G, 5'UTR-840T→G and 5'UTR-467G→C variants while they were homozygous wild type for the H63D variant. Proband 2 was heterozygous for the 5'UTR-1206C→G, 5'UTR-467G→C and 5'UTR-840T→G variants and homozygous wild type for the H63D variant. The haplotype observed in the two probands was not present in any of the extended family members.

A haplotype similar to that identified in the probands was observed in individual II:5. The individual was heterozygous for the 5'UTR-1206C→G and 5'UTR-467G→C variants and homozygous wild type for the H63D variant. This family member was also homozygous wild type for the novel 5'UTR-840T→G variant while the probands were heterozygous for this variant. This indicates that this novel variant could possibly be associated with the iron overload phenotype.

In silico analysis of the *HFE* promoter revealed that in the presence of the novel 5'UTR-840T→G variant a putative PU.1 TFBS is abolished. This transcription factor binding site was also conserved within the *HFE* promoter region (see Table 3.5).

Osteoporosis develops in many HH patients with *HFE* mutations but the mechanism involved in the development of osteoporosis has not been fully elucidated. The PU.1 transcription factor plays an important role in macrophage differentiation (Tondravi *et al*, 1997) and therefore modulates osteoclast development as osteoclasts are derived from these cells (Udagawa *et al*, 1990). The PU.1 transcription factor has been associated with osteoporosis development. Mice lacking a functional *PU.1* gene developed osteoporosis as no

macrophages or osteoclasts were present (reviewed by Teitelbaum, 2000). As mentioned previously proband 1 presented with osteoporotic symptoms. Although the regulatory effect that PU.1 has on the expression of *HFE* has not yet been elucidated, it is possible that PU.1 and HFE function in concert and that by disrupting their interaction, the novel 5'UTR-840T→G variant could be responsible for the osteoporotic symptoms that developed.

PU.1 has also been established as a regulator of T-cell development. PU.1 expression is tightly regulated during immune cell development and dysregulation of its expression may lead to immune disorders such as diabetes and arthritis (Fang, University of Missouri, Columbia, 2006) (available online: <http://www.dana.org/grants/imaging/detail.aspx?id=4430>). Proband 1 presented with non-insulin-dependent diabetes at diagnosis and later developed arthritis in his hips, knees and hands. Furthermore, patients with type 1 HH (*HFE*-associated) may also develop arthritis and diabetes. Hepcidin plays an important role in the immune response and by regulating the amount of available iron, regulates inflammation and infection (reviewed by Vyoral and Petrák, 2005). The 5'UTR-840T→G *HFE* variant as well as the 5'UTR-335G→T *HAMP* variant disrupt a putative PU.1 TFBS. *HFE* and *HAMP* may function in the same immune cell regulatory pathway as PU.1 and by disrupting the interaction between PU.1 and these peptides; these two variants could be contributing to the development of the arthritis and diabetes observed in proband 1.

The hepcidin levels of HH patients homozygous for the C282Y *HFE* variant are very low (Bridle *et al*, 2003). Hepcidin expression is also reduced in the *Hfe*-knockout mouse model (Ahmad *et al*, 2002). Furthermore, virtually no hepcidin is detected in the urine of individuals with *HJV* mutations. These findings indicate that *HFE* and *HJV* may function in the same regulatory pathway and regulate the expression of hepcidin. It has been shown that *HJV* positively regulates the expression of the *HAMP* gene *via* the BMP signalling pathway (Babitt *et al*, 2006). *HFE* could possibly also be involved in this regulatory pathway but its potential role needs to be investigated further.

The *HFE* variant could also be modulating the iron overload phenotype by providing a protective effect against the severe iron overload that is usually associated with *HAMP* mutations (refer to section 1.1.2). As can be seen in Table 3.5 a putative specificity protein 1 (SP1) TFBS is created in the presence of the 5'UTR-840T→G variant. SP1 is a ubiquitous transcription factor and has been implicated in the regulation of many genes. Although its role

in the regulation of *HFE* transcription has not yet been elucidated, SP1 binding sites have been identified within the *HFE* promoter (Mura *et al*, 2004). By creating an additional SP1 binding site this variant could increase HFE expression. Although the exact function of the HFE peptide in iron metabolism still remains unclear, it does seem to be involved in the regulation of the amount of iron absorbed from the gut lumen. Excess iron is absorbed in individuals with mutations in the *HFE* gene, such as C282Y, which result in diminished HFE availability. By potentially increasing the expression of HFE, the 5'UTR-840T→G variant could result in diminished iron absorption. This may counteract, but not completely make up for, the effects of the *HAMP* variant and may therefore be responsible for the less severe iron overload phenotype observed in the two Indian probands. Functional analysis of this variant needs to be performed to determine if the 5'UTR-840T→G variant is indeed a gain-of-function variant.

***HAMP* gene**

The Indian family analysed was highly consanguineous and only two family members were identified who presented with the HH phenotype. Furthermore, the probands are siblings and are the product of a first cousin relationship. This indicates that the HH phenotype is most likely inherited in an autosomal recessive manner and that a homozygous variant in any of the six genes would be a likely candidate as the causative variant.

A single previously identified variant was identified in the promoter region of the *HAMP* gene (5'UTR-335G→T) (HapMap; refSNP ID: rs3817623). This variant was identified in the homozygous state in both probands but none of the extended family members presented with the homozygous genotype (see Figure 3.5). This variant was observed in the heterozygous state in 16 family members. All heterozygous family members presented with iron parameters within the normal range except individual III:7 who was iron deficient (SF level: 15 µg/l) and individual III:10 who presented with a slightly elevated SF level (213 µg/l). The heterozygous genotype does not seem to be associated with iron overload as heterozygous individuals with similar ages to the probands were identified (II:2, II:4, II:5 and II:6) and presented with normal iron parameters. The polymorphic allele has not been detected in the European or sub-Saharan African populations but has been detected in the Asian population (0.034-0.044) (HapMap project). Furthermore, the variant genotype has been reported in the heterozygous state in the Asian population with a genotypic frequency of 0.068-0.089, however, the

homozygous form has not been documented. It should be noted that this Asian population does not include Asian Indians but individuals from the Japanese and Han Chinese populations.

In silico analysis of this promoter variant revealed that in the presence of the polymorphic allele three putative TFBS are abolished (see Table 3.6). These include the interleukin-4 (IL4), vitamin D3 receptor (VDR) and spleen focus forming virus proviral integrating oncogene 1 (PU.1). Further analysis employing the rVISTA program did show that these three transcription factors are conserved within the *HAMP* promoter but did not confirm that they were abolished in the presence of the variant allele.

PU.1 is an ETS-domain transcription factor. It plays an essential role in the development of myeloid and B-lymphoid cells. Tondravi *et al* (1997) demonstrated that PU.1 mRNA gradually increased as bone marrow macrophages differentiated into osteoclasts. They also noted that both osteoclast and macrophage development did not occur in PU.1 deficient mice and that these mice ultimately developed osteoporosis. Osteoporosis is a disease characterised by generalised low bone mass and microarchitectural deterioration of bone tissue. The bones of osteoporotic patients are more fragile and there is an increased susceptibility to fracture (reviewed by Rizzoli *et al*, 2001). Polymorphisms within VDR have been associated with a decreased bone mineral density and osteoporosis (Morrison *et al*, 1992; Gennari *et al*, 1998; Ferrari *et al*, 1999). Hepcidin could be functioning in the same pathway as the VDR and PU.1 transcription factors and regulating osteoclast development and differentiation. Although not all HH patients develop osteoporosis, it is interesting to note that both of the transcription factors that are abolished by the 5'UTR-335G→T *HAMP* variant have been implicated in the development of this disease. Further research is necessary to determine the role that this variant may play in the development of osteoporosis in HH patients.

Hepcidin is an essential peptide involved in the regulation of iron absorption in the intestine and iron recycling from macrophages. It has also been proposed as the key regulator of anaemia of inflammation (Fleming and Sly, 2001; Weinstein *et al*, 2002). Hepcidin expression induced in the presence of IL6 but not IL1- α or tumour necrosis factor (TNF)- α and therefore seems to be regulated by a type 2 acute phase response (Nemeth *et al*, 2003). The entire mechanism of hepcidin regulation has not as yet been elucidated. IL4 is a type two cytokine and may be involved in the regulation of hepcidin expression. Vogel and colleagues

(2002) attempted to determine the role mutated VDR plays in modifying the autoimmune response. The VDR binds to the active form of vitamin D (1,25-dihydroxyvitamin D₃) and translocates to cell nuclei where it regulates a multitude of biological effects (Baker *et al*, 1988). The authors noted that the binding of 1,25-dihydroxyvitamin D₃ produced various effects including decreasing the expression of type 1 cytokines and increasing the expression of IL4 (Vogel *et al*, 2002). These results indicate that VDR and IL4 may play a role in the regulation of hepcidin. In the presence of the variant allele both of these putative TFBS are removed and this could ultimately influence the expression of the *HAMP* gene. Decreased expression of hepcidin has been implicated in the development of the severe iron overload disorder JH (Roetto *et al*, 2004; Matthes *et al*, 2004). The role that VDR and IL4 play in *HAMP* gene expression needs to be investigated further.

Functional analysis of this variant is currently underway. Transcription activity from the mutated and wild type *HAMP* promoter is being investigated. Luciferase reporter constructs transfected with the mutated and wild type *HAMP* promoters have been developed to determine if this variant affects *HAMP* mRNA expression. Preliminary results indicate that mRNA expression from the *HAMP* promoter is strongly impeded in the presence of the 5'UTR-335G→T variant. These preliminary findings provide supporting evidence that we have identified the variant causing HH within this Indian family.

Genotyping of this variant in an unaffected, general population matched control cohort was not performed. The family under investigation was a first generation South African Indian family and screening of the South African Indian population may not be a true reflection of the prevalence of this variant in the Asian Indian population. Analysis and comparison of this variant in the Asian Indian population as well as the South African Indian population is necessary to determine if this variant is truly associated with HH in these general populations or if it is a private variant only causing HH in this family.

***SLC40A1* gene**

Mutation analysis of the *SLC40A1* promoter and coding regions revealed five previously identified variants (5'UTR-1355G→C, 5'UTR-750G→A, 5'UTR-593C→T, 5'UTR-501T→C and V221). The previously described CGG microsatellite within the *SLC40A1* promoter (Lee *et al*, 2001) was also identified with either 7 or 8 repeats.

As can be seen in Figure 3.6 only three different haplotypes were present in the second generation family members. As mentioned previously this could occur as the probands' parents were first cousins. The two probands presented with different haplotypes for this gene. Analysis of unaffected family members with similar ages to the probands revealed that individuals II:2 and II:6 presented with the same haplotypes as proband 1 and individual II:5 presented with the same haplotypes as proband 2. As none of these family members was iron loaded, the *SLC40A1* variants do not seem to be associated with the iron overload phenotype and may be common polymorphisms within this Indian family.

***CYBRDI* gene**

Ten previously described (5'UTR-1844C→G, 5'UTR-1834G→A, 5'UTR-1813C→T, 5'UTR-1459T→C, 5'UTR-624G→A, 5'UTR-238A→G, 5'UTR-167C→G, 5'UTR-163G→A, IVS2+8T→C and S266N) and two novel (5'UTR-1452T→C and 5'UTR-1272T→C) variants were identified in the *CYBRDI* gene. A repeat was also observed in the 5'UTR [G(T)₈G(T)₆G(T)_nG(T)₉] where n represents either zero or six repeats.

Haplotype analysis revealed that the two probands presented with exactly the same haplotypes (haplotype 1 and haplotype 2) (see Figure 3.11). The same haplotypes were also observed in a 63-year-old male family member (II:2) who presented with normal iron parameters. This indicates that all of the variants identified within the *CYBRDI* promoter and coding regions do not seem to be associated with the iron overload phenotype.

***HJV* gene**

Two previously described (5'UTR-1406C→A and 5'UTR-542A→G) and two novel variants (5'UTR-534G→T and 5'UTR-530G→T) were identified within the *HJV* gene promoter region. An AAGG variable number tandem repeat (VNTR) or microsatellite was also identified in the promoter region with 11 to 13 repeats (allele 1 = 11, allele 2 = 12, allele 3 = 13).

The haplotypes constructed for the *HJV* gene were identical in the two affected probands (see Figure 3.14). The same haplotype was identified in two of the probands' siblings (II:5 and II:7). These two individuals had similar ages to the two probands but did not display

symptoms of iron overload as their iron parameters were within the normal range. These results indicate that the *HJV* gene variants identified in the Indian family are not associated with the iron overload phenotype.

To conclude, mutation analysis of the *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* and *HJV* genes revealed many variants in the Indian family under investigation. Of the variants identified, the homozygous 5'UTR-335G→T variant identified in the *HAMP* promoter seems to be the most likely candidate responsible for the iron overload phenotype in the two Indian probands. *HAMP* variants are associated with the severe iron overload phenotype observed in JH. The two probands in this Indian family did not present with the severe iron overload phenotype but rather a milder form similar to classic or Type 1 HH. Variants within the *HMOX1* (5'UTR-495A→T) and *HFE* (5'UTR-840T→G) genes could all be modulating the effect of the homozygous *HAMP* variant and producing the less severe iron overload observed. These variants may also, in part, be causing some of the symptoms characteristic of HH such as liver cell damage, liver cirrhosis and osteoporosis. The remainder of the single nucleotide substitutions identified in the six genes seem to represent common polymorphisms within this Indian family. Analysis within the general Indian population is required to determine if they are SNPs within this population. It should also be considered that the presence of mutations/variants within the unaffected family members could be providing a protective effect. This possibility needs to be investigated further.

CHAPTER FOUR

CONCLUSIONS AND FUTURE PROSPECTS

4. Conclusions and Future Prospects

Hereditary haemochromatosis (HH) is one of the most common genetic disorders in people of northern European descent. Iron homeostasis is disrupted in patients with HH and this results in uncontrolled iron absorption from the gut lumen. The excess iron is stored in various tissues where it may damage cell membranes, proteins and DNA. Treatment of the disease by regular phlebotomy is cheap and relatively non-invasive. If the disease progresses undiagnosed and untreated it can result in the development of diabetes mellitus, skin hyperpigmentation, liver cirrhosis and primary liver cancer. Although well characterised in the Caucasian population, iron overload in non-Caucasians is not well defined and the gene aberrations associated with non-Caucasian iron overload still remain to be identified. Iron overload and specifically, hereditary haemochromatosis, is predicted to be rare and not well documented within the Asian Indian population. Various research groups have determined that iron overload in Indians is not associated with the C282Y or H63D mutations in *HFE* and is of the non-*HFE* type.

The aim of this study was to identify known and novel variants within iron regulatory genes that were contributing to the *HFE*-associated HH phenotype observed in two probands from an Indian family. Six genes involved in iron regulation were screened including the *HMOX1* gene, *HFE* gene, *HAMP* gene, *SLC40A1* gene, *CYBRD1* gene and the *HJV* gene. Mutation analysis of these genes in the two Indian probands and 23 of their unaffected family members revealed 24 previously described single nucleotide variants, five novel single nucleotide variants, one previously described microsatellite within the *SLC40A1* gene and two novel repeats, one in the 5'UTR of the *CYBRD1* gene and another in the 5'UTR of the *HJV* gene.

As mentioned previously it has been reported that haemochromatosis in the Indian population is not associated with the common *HFE* gene mutations C282Y and H63D. Both of these mutations were not present in the two probands. The C282Y mutation was not observed in any of the unaffected family members but the H63D variant was present in the heterozygous state in four family members who presented with normal iron parameters. Therefore the C282Y and H63D mutations are not playing a role in iron loading within this Indian family. These results corroborate the findings of other researchers who have analysed these mutations

in the Indian population and reported that iron overload on the Indian subcontinent is not associated with the two common *HFE* mutations (Garewal *et al*, 2005; Dhillon *et al*, 2007).

Haplotype analysis was performed for the variants identified in each of the genes investigated. The probands' haplotypes constructed for the *SLC40A1*, *CYBRD1* and *HJV* variants were also observed in unaffected family members, indicating that the variants in these three genes are not associated with the HH phenotype observed in the two probands. The prevalence of these variants within the probands and unaffected family members indicates that these variants are common polymorphisms in the Indian family. Further analysis in a larger study cohort from the general Indian population is necessary to confirm if these variants are polymorphisms in the general Indian population rather than being common only in this highly consanguineous family.

The previously described 5'UTR-335G→T variant identified in the *HAMP* gene was observed in the homozygous state in both affected probands. This variant was observed in the heterozygous state in 16 unaffected family members. The Indian family under investigation is a highly consanguineous one and the two probands are the product of a consanguineous relationship. The fact that only two individuals are afflicted with HH increases the likelihood that the iron overload disorder is inherited in an autosomal recessive manner in this family. Therefore, a homozygous variant identified in the two probands, but absent in the homozygous variant form from the unaffected family members, would be a likely candidate for causing the disease phenotype.

Preliminary results from functional analysis of this *HAMP* promoter variant have indicated that expression from the mutated *HAMP* promoter is greatly inhibited. This preliminary data supplies supporting evidence that the 5'UTR-335G→T *HAMP* variant is responsible for the iron overload phenotype observed in the two Indian probands.

Mutations in the *HAMP* gene have previously been associated with the severe iron overload observed in juvenile haemochromatosis patients. Patients with JH exhibit symptoms more severe than those with Type 1 HH. The two probands identified in this study do not exhibit symptoms of JH but of the less severe Type 1 or *HFE*-associated HH. A possible explanation for the less severe HH phenotype observed in the two probands is that variations in other iron regulatory genes are involved in the pathogenesis of HH. Variants identified in other genes

may be modifying the effects of the homozygous *HAMP* promoter variant resulting in the less severe phenotype.

Haplotype analysis of the variants identified in the *HMOX1* and *HFE* genes indicated that variants unique to the haplotypes observed in the two probands might be modulating the iron overload phenotype. These variants include: 5'UTR-495A→T (*HMOX1*) and 5'UTR-840T→G (*HFE*). These variants may also be responsible for the symptoms that manifested within the two probands.

Future researchers should investigate the role that the *HMOX1* and *HFE* gene variants play in the modification of the HH phenotype. Multiple families and a larger group of unrelated affected Indian HH patients should be analysed to either corroborate or refute the findings of this study. Animal models should also be developed with knock-in of the *HAMP* variant in conjunction with the *HMOX1* and *HFE* variants to determine how they affect iron homeostasis.

A limitation of the present study was that the study cohort only included members from a single highly consanguineous family. The prevalence of the variants identified is not a good indication of the prevalence of these variants in the Asian Indian population as they would understandably be more common in this family. In this family the two probands were siblings and there were no affected family members in the successive generation. This limited the efficiency of haplotype analysis, as it could not be determined for certain which was the variant haplotype. A further limitation was that haplotype analysis was incomplete for many of the third generation family members as the haplotypes of only one parent was established. Future studies should include the analysis of various multi-generation families with affected family members in different generations to improve the efficacy of haplotype analysis.

Another limitation of this study was the effectiveness of the screening technique. Although many known and novel variants were identified there is a possibility that additional variants were overlooked. Evidence to support this is provided by the fact that only a single variant was identified in the *HAMP* gene. Furthermore, genotyping of some of the variants was inconclusive for various family members following HEX-SSCP analysis and therefore haplotype analysis was incomplete for these individuals. Single-strand conformation polymorphism (SSCP) analysis is predicted to exhibit a specificity of 70-100% when DNA

fragments of 130-250 bp are analysed (Xiao and Oefner, 1992; Bonner and Ballard, 1999). The size of all the DNA fragments analysed in the present study, with the exception of the *HMOX1* exon 3 fragment amplified with primer set 3A, exceeded 250 bp. Although the variant detection rate of this technique was improved by the combined analysis of heteroduplexes (HEX-SSCP analysis), the size of the DNA fragments could influence the efficacy of this screening technique. Future research should focus on either optimising the HEX-SSCP technique for each DNA fragment analysed or should employ a more sensitive screening technique. Larger DNA fragments (198-732 bp) can be analysed using denaturing high-performance liquid chromatography (dHPLC) analysis and the sensitivity of this technique is reportedly 92-100% (Underhill *et al*, 1997). This technique is superior to SSCP analysis in terms of its sensitivity and efficiency. More costly screening techniques are also available such as bi-directional semi-automated DNA sequencing analysis and DNA chip technology. Bi-directional semi-automated DNA sequencing allows for the identification of all variants within a DNA fragment and is considered to be the most effective screening technique available (Kristensen *et al*, 2001). DNA chips allow for the detection of a multitude of known variants in different genes. DNA chips could be constructed for known variants implicated in the pathogenesis of HH and assist in the simultaneous detection of alleles associated with the disease. Bi-directional semi-automated DNA sequencing and chip technology is more effective than SSCP but also more costly and therefore are not viable options in all research laboratories.

Another limitation was observed after employing semi-automated DNA sequencing analysis for the genotyping of some variants in the extended family members. Various individuals presented with genotypes that were ambiguous and did not conform to the haplotypes present in the family. The genotypes of these individuals were subsequently omitted from the haplotype analysis as the ambiguity could not be explained by non-paternity or recombination. The sequencing electropherograms for these individuals were double-checked by the researcher and supervisor and the same results were obtained. A possible explanation for the observed discrepancy is that the samples were swapped in the sequencing laboratory and that the incorrect sample was sequenced. Samples should always be double-checked and labelled carefully to prevent this from occurring.

PCR amplification was not optimised for fragment 5 of the *HMOX1* promoter, fragment 2 of the *HAMP* promoter and fragments 2 and 5 of the *HJV* promoter. Therefore the mutation

analysis of these promoter regions was incomplete. Future researchers should attempt to optimise the PCR technique for these fragments or should redesign primers flanking the relative regions. Mutation analysis of these fragments could possibly reveal additional variants in the promoters of these three genes that may be associated with the iron overload phenotype. Genotyping of the IVS2+8T→C *CYBRDI* intronic variant was incomplete as HEX-SSCP results were unclear. The genotypes of the family members for this variant need to be clarified employing semi-automated DNA sequencing analysis.

HH is rare in the Indian population and the causative gene has not yet been elucidated. The homozygous 5'UTR-335G→T *HAMP* variant seems to be causing HH in the Indian family investigated in this study. Further analysis within the general Asian Indian population is necessary to determine whether this variant is associated with HH in other Indian patients or if it is a private variant which is only present in this highly consanguineous family. Future research should also focus on identifying any genetic factors that could be modifying the expression of this variant. The identification of the involvement of the homozygous 5'UTR-335G→T *HAMP* variant in the pathogenesis of HH in these two Indian probands may assist in the elucidation of the elusive iron loading gene in the Asian Indian population.

CHAPTER FIVE

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5.2 Electronic-Database Information

Alternative Splice Site Predictor: <http://www.es.embnet.org/~mwang/assp.html>

Ensembl: <http://www.ensembl.org>

ESEfinder: <http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi>

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HapMap (Online): <http://www.hapmap.org>

Online Mendelian Inheritance in ManTM (OMIMTM):
<http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM>

PATCH: <http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi>

Primer3: http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi,2002

rVISTA: <http://genome.lbl.gov/vista/rvista/submit.shtml>

TRANSFAC ®7 database: <http://www.gene-regulation.com/pub/databases.html#transfac>

APPENDIX 1: LIST OF CHEMICALS/REAGENTS USED IN THIS STUDY AND THEIR SUPPLIERS.

Chemical/Reagent	Supplier
Acrylamide	Sigma-Aldrich
Agarose	Laboratory Specialist Services
APS	Merck
Bisacrylamide	Sigma-Aldrich
Bromophenol blue	Seabreeze Suppliers
Cresol red	Merck
ddH ₂ O	Adcock-Ingram
dNTPs (dATP, dTTP, dCTP, dGTP)	Fermentas
EDTA	Seabreeze Suppliers
EtBr	Fluka
EtOH	Seabreeze Suppliers
Formamide (De-ionised)	Merck
H ₃ BO ₃	Seabreeze Suppliers
KCl	Roche Diagnostics
KHCO ₃	Merck
KH ₂ PO ₄	Seabreeze Suppliers
NaCl	Fluka
Na ₂ HPO ₄	Seabreeze Suppliers
Urea	Sigma-Aldrich
NH ₄ Cl	Seabreeze Suppliers
PBS	Fluka
Proteinase K	Roche Diagnostics
SDS	Seabreeze Suppliers
Sucrose	Seabreeze Suppliers
<i>Taq</i> polymerase	Fermentas
TEMED	Fluka
Tris-HCl	Fluka
Xylene cyanol	Fluka

APPENDIX 2: PROMOTER AND CODING REGIONS OF *HMOX1*, *HFE*, *HAMP*, *SLC40A1*, *CYBRD1* AND *HJV* GENES INDICATING PRIMER BINDING POSITIONS AND VARIANTS IDENTIFIED IN THIS STUDY.

Promoter (5' to 3') and coding regions (5' to 3') of the respective genes were selected from Ensembl and GenAtlas. The primers designed for the analysis of the promoter regions are highlighted in various colours (described below). Primers designed for the analysis of the exons of each gene are indicated using various text colours. Arrows flank the primers with arrowheads indicating the orientation of each primer. The translation initiation site (ATG) and the stop codon (TGA, TAA or TAG) are indicated in bold red text. Variation detected in the current study is highlighted in grey and the superscript number indicates the nucleotide position (described below). The 5'UTR and introns of genes are indicated in lowercase text while the mRNA encoding regions (transcribed regions) are indicated in uppercase. The bold mRNA regions are those that form part of the translated peptide (translated). Variants identified in the present study are highlighted in grey and the superscript number indicates the nucleotide position, which is indicated in the key.

HMOX1 promoter sequence

[ENSG00000100292 (Ensembl)]

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acagggctccctatggtgcccaggccagtctcgaactcaaagcaatcttcccacctcgactgggctcaaagcgc
tcttcccacctcaacctcccaaagtactgggactacaggtgtgagctaccatgccaggcctgaaagccatcttaa
aaaaaaaaatcttagaatgagaatcacagttatgggaaaggactgtatgaatcatctggccattcggtttgtcct
ctgggttaccacagtgacctatctcccccgagttctaaggagtccacctcatgcagaattgattcaataggcga
tcagcaagggccagctctgctctgggcctgagcaggcactgagtataagtacagacctgaatgtgcctggaagag
tgtcccacgcattccagcaggggaagcagtttgatgacaggtgtcccagtcaggcggataccagggtgctgcccag
agtggtggaggaggcaggcggggacttagtctcctccctgggttggacactggcatcctgctttatgtgtgacac
cactgcacccctctgagcctcggtttccccatctgtaaaatagaagcgatctaccctcacagggtcagttgtaggg
atgaaccatgaaaatactagagttctctgttttttgacaggaactcaaaaaacagatcctaaatgtacatttaaag
agggctgtgaggaggcaagcagtcagcagaggattccagcaggtgacatttttagggagctggagacagcagagcct
ggggttgctaagttcctgatgttgcccaccaggctat1tgctctgagcagcgctgcctcccagctttctggaacc
ttctgggacgcctggggtgcatcaagtcccaaggggacagggagcagaaggggggctctggaaggagcaaaatc
acaccagagcctgcagcttctcagatttccctaaagggtttgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtatg
tgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtatg
actgctcctctccacccacactggccccggggcgggctgggcgcgggcccctgcccgggtgttgcaacgcccgccca
gaaagtgggcatcagctgttccgcctggcccacgtgacctgcgagcataaatgtgaccggccgcggtccggca

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gtcaacgcctgcctcctctcgagcgtcctcagcgcagccgcccgcggagccagcacgaacgagcccagcacc
 ggccgg**ATG**gagcgtccgcaaccgacagggcaagcgcggggcgcgggacgcgggacggggc**ctttctctccaa**
ccctgcttgctcctagccccacccgggacactgccacacagcgcagagcccaggagccagaaacttgggctc

***HMOX1* coding regions**

[NM_002133 (GenAtlas)]

Exon 1

gccagactttgtttcccaagggcatatgactgctcctctccacccacactggcccggggcgggctgggcgcg
gcccctgcgggtgttgcaacgcccggccagaaagtgggcatcagctgttcgcctggcccacgtgac**ccgcccgag**
cataaatgtgaccggccgcgggtccggcagTCAACGCCTGCCTCCTCTCGAGCGTCTCAGCGCAGCCGCCGCC
 GCGGAGCCAGCACGAACGAGCCAGCACCCGGCCGG**ATG**GAGCGTCCGCAACCCGACAGgcaagcgcggggcgcg
 ggacgcgggacgggcgctttctctcccaaccctgcttgcgtcctagcccacccgggacactgccacacagc
 acagagcccaggagccagaaacttgggctctggagttaggaggtgcggg**gtttctgatcctgcctgtgc**ccgtagg
 gtagtggaggag

Exon 2

gggattacagcgtgagccaccgtgccagccacaaggctgcatcttaagcgattgagaacgtggcctgaatga
ggatgggagtctctgaaggcctgccacaggtgggaggtcagcagttgggaaggacccacccc**cagccagct**
ttgtgttcacctttccatttctcctcctcag**CATGCCCCAGGATTTGTCAGAGGCCCTGAAGGAGGCCACCAAGGA**
GGTGACACCCAGGCAGAGAATGCTGAGTTCATGAGGAACTTTTCAGAAGGGCCAGGTGACCCGAGACGGCTTCAA
 Ggtat²gtggcttggggactagccctggtggaggggtgtggcaggtgtgggtggacc**caaggctcagaccagtg**
gtttaagtgggatgctgagggaccagatgggcatgtccaatagaatcatcttaaaaatgatgacactgaggctc
 agagaggggaaggtgagttacccaaggtcacac

Exon 3

Tcctcttgtaaaaaccctctggctgctgtgtgaagaggattgtagcaggggtggcagaaggagttagagccca
 gctgcgaagtgaggagggcctttccaaaggcag**tagtggacgggacggacaga**gggtgggggtcttctatgtggct
 ggcggcctgac³tgctcactctgctttcag**CTGGTGATGGCCTCCCTGTACCACATCTATGTGGCCCTGGAGGA**
GGAGATTGAGCGCAACAAGGAGAGCCCAGTCTTTCGCCCTGTCTACTTCCC**AGAAGAGCTGCACCGCAAGG**CTGC
 CCTGGAGCAGGACCTGGCCTTCTGGTACGGGCCCCGCTGGCAGGAGGTCATCCCCTACACACCAGCCATGCAGCG
 CTATGTGAAGCGGCTCCACGAGGTGGGGCGCACAGAGCCCAGCTGCTGGTGGCCACGCCTACACCCGCTACCT
 GGGTGACCTGTCTGGGGCCAGGTGCTCAAAAAGATTGCCAGAAAGCCCTGGACCTGCCAGCTCTGGCGAGGG
CCTGGCCTTCTTCACCTTCCCAACATT
GCCTGGCCTTCTTCACCTTC
GCCAGTGCCACCAAGTTCAAGCAGCTCTACCGCTCCCGCATGAACTCCCTGGAGATGACTCCCGCAGTCAGGCAG
AGGGTGATAGAAGAGGCCAAGACTGCGTTCTGCTCAACATCCAGgtgagggctcgggcagcctggggcagcctct
 gcctcccccggttgttctccaagggacccttctcattgtagggaggggtgctataggtcatggttaacacaggg
aa**ccagagttccagcaactgcca**cttactagctgggtgatcttgggcaaatgccttcatctctctgtacctcagtt

HFE promoter sequence

[ENSG0000010704 (Ensembl)]

aatttgctagatatgtaaa **ggtttggagcaaatcaggtg** tattaatattattaatattgtttgaaatgtcctaagg
 caataattcccaacttcggtgagggagaggaaagcttttaaaatcccattgcccaggtggcatcccatactgt
 tactgggaattatgcattgggatggatcctttaaccgaggagattattatagc **eggagctctgaa**¹ **cagcaat**¹ c
 tcagttcttgtgatagtgagcaagaactacaaactaacacccaaaatgcaagcttaagcaagtttattgaaagc
 acaataatacactctgagggacagcgggcttattt **ctgcaagtgaactcagcac** tctttacagagctcaaggt
 gcttttatgggggttgtggggaggagt **tgaggtttgggctgtatctg** agtgacaggatgatgttatttgattgaa
 gtgtatagctatacaatctaaaattaa **ctgtgcatggcttacctat** aatttgtaagaaaagcctcccagggg
 tgggggggcaaaactgtatgtaaattctattataatgatggcatgatgaacttgggg² **gaacttgaagacagggc**
 ttttgtgttggg **catgtgccaccttagggaat** tccacctgtaccctcctttctctttctc **caggatatttt**
ggccacagac tttatcataaactccatcccttaggggtggcattagggtagtcttgggctgaatttaggtgggccc
 agtggtgtct **cttagtgacagcctttccgct** ctct **ctgtcatccccctcccaact** gctaatgtctaactacctaac
 aattaccattaaatcagtg **gtgtctggggttaggagcag** gcctcaatatgtttaatcattctccagataatccc
gtgtctggggttaggagcag
 aactactgtaaagtttgtgaaacacttgtcagataattcaatta **tgaaggtgtggaag**³ **gtgtt** tcagtaggatc
 taattggttaatgttatgacttaattaatttgaatcaaaaaacaaaatgaaaagctttatatttctaagtcaaa
 taagacataagttgggtctaaggttgagataaaaatttttaaagtatgattgaattttgaaaatcataaatattta
 aatatctaaagttcagatcagaa **cattgcaagctactttccc** caatcaacaacacccttcaggatttaaaaac
 caagggggacactggatc **acctagtgtttcacaagcag** gtacctctgctgtagggagagagagaactaaagtct
 gaaagacctgttgcctttccaccaggaagtt **ttactgggcatctcctgag** cctaggcaatagctgtagggtgactt
 ctggagccatccccggtttccccgccccccaaaagaagcggagatttaacggggacgtgcggccagagctggggaa
atgggcccgcgagccagggcggcgcttctcctcctgatgcttttgcagaccgggctctgcaggggcgct **ttgctg**
cgtgagtcaggg gctgcgggcgaactaggggcgcggggggtggaaaaatcgaaactagctttttctttgctg
 ttgggagtttgctaactttggaggacctgctcaacctatccgcaagcc

HFE coding regions

[NM_000410 (GenAtlas)]

Exon 1

aaaaagctttatatttctaagtc aaataagacataagttgggtctaaggttgagataaaaatttttaaagtatgat
 tgaattttgaaaatcataaatattttaaataatctaaagttcagatcagaacattgcaagctactttcccacatca
 acaacacccttcaggatttaaaaaccaagGGGGACACTGGATCACCTAGTGTTCACAAGCAGGTACCTTCTGC
 TGTAGGAGAGAGAGAACTAAAGTTCTGAAAGACCTGTTGCTTTTCACCAGGAAGTT**TFACTGGGCATCTCCTGAG**
CCTAGGCAATAGCTGTAGGGTGACTTCTGGAGCCATCCCCGTTTCCCCGCCCCCAAAAGAAGCGGAGATTTAAC
 GGGGACGTGCGGCCAGAGCTGGGGAA**ATGGGCCCGGAGCCAGGCCGGCGCTTCTCCTCCTGATGCTTTTGCAGA**
CCGCGGTCCTGCAGGGGCGCTTGTCTGCgtgagtcaggggctgcgggcgaactaggggcgcgggc **ggggtggaaa**
aatcgaaactagctttttctttgctgtgggagtttgcctaactttggaggacctgctcaacctatccgcaagcc
 cctctccctactttctgctccagacccctgagggagtgctaccactgaactgca

Exon 2

aagcacacaaggaaagagcaccaggactgtcatatggaagaaagacaggactgcaactcacccttcacaaaatg
 aggaccagacacagctgatggatgatgagttgatgcaggtgtgtggagcctcaacatcctgctcccctcctactaca
 catggttaaggcctgttgcctgtctccagGTTACACTCTCTGCACTACCTCTTCATGGGTGCCTCAGAGCAGG
 ACCTTGGTCTTTCTTGTGTTTGAAGCTTTGGGCTACGTGGATGACCAGCTGTTTCGTGTTCTATGATC⁴ATGAGAGT
 CGCCGTGTGGAGCCCCGAACCTCCATGGGTTTCCAGTAGAATTTCAAGCCAGATGTGGCTGCAGCTGAGTCAGAGT
 CTGAAAGGGTGGGATCACATGTTCACTGTTGACTTCTGGACTATTATGGAAAA⁴TCACAACCACAGCAAGGgtatg
 tggagagggggcctcaccttctgaggttgtcagagcttttcatcttttcatgcatcttgaaggaaacagctgga
 agtctgaggtcttgtgggagcaggaagaggaaggaatttgcttctgagatcatttggctcttgggatggtg
 gaaatagggacctattccttttggtt

Exon 3

aggttgtcagagcttttcatcttttcatgcatcttgaaggaaacagctggaagtctgaggtcttgtgggagcag
 gaagaggaaggaatttgcttctgagatcatttggctcttggggatggtggaataaggacctattccttttggt
 tgcagttaacaaggctggggatttttccagAGTCCACACCCTGCAGGTCATCCTGGGCTGTGAAATGCAAGAAG
 ACAACAGTACCGAGGGCTACTGGAAGTACGGGTATGATGGGCAGGACCACCTTGAATTCTGCCCTGACACACTGG
 ATTGGAGAGCAGCAGAACCAGGGCCTGGCCCACCAAGCTGGAGTGGGAAAGGCACAAGATTCTGGGCCAGGCAGA
 ACAGGGCTACTGAGAGGGACTGCCCTGCACAGCTGCAGCAGTTGCTGGAGCTGGGAGAGGTGTTTTGGACC
 AACAAGgtatggtggaacacacttctgcccctatactctagtggcagagtggaggaggttgcagggcacggaat
 ccctggttggagtttcagaggtggctgaggtgtgtgcctctccaaattctgggaagggactttctcaatcctag
 agtctctaccttataattgagatgtatgagacagcc

Exon 4

ctccaagtgcactgtgttagagtcctaatcttaggacacaaaatggtgtctctcctgtagcttgttttttctga
 aaaggtatcttctcctccaacctatagaaggaagtgaaagttccagcttctctggcaagggtaaacagatccc
 ctctcctcactccttctcttctctgtcaagTGCCCTCTTTGGTGAAGGTGACACATCATGTGACCTCTTCAGTGA
 CCACTCTACGGTGTCTGGGCTTGAACCTACTACCCCAAGACATCACCATGAAGTGGCTGAAGGATAAGCAGCCAA
 TGGATGCCAAGGAGTTTGAACCTAAAGACGTATTGCCCAATGGGGATGGGACCTACCAGGGCTGGATAACCTTGG
 CTGTACCCCTGGGGAAGAGCAGAGATATACGTGCCAGGTGGAGCACCCAGGCCTGGATCAGCCCTCATTTGTA
 TCTGGGgtatgtgactgatgagagccaggagctgagaaaatctattggggggttgagaggaggtgcctgagaggta
 attatggcagtgagatgaggatctgctctttgttagggggtgggctgaggggtggcaatcaaaggctttaacttgc
 tttttctgttttagagccctcaccgtctggcacct

Exon 5

cagcccctcattgtgatctggggtatgtgactgatgagagccaggagctgagaaaatctattgggggttgagagg
 agtgcctgaggaggaattatggcagtgagatgaggatctgctctttgttagggggtgggctgaggggtggcaatc
 aaagctttaacttgccttttctgttttagAGCCCTCACCGTCTGGCACCTAGTCATTGGAGTCATCAGTGGAA
 TTGCTGTTTTTGTCTCATCTTGTTCATTGGAATTTTGTTCATAATATTAAGGAAGAGGCAGGGTTCAAgtgagt
 aggaacaagggggaagtctcttagtaacctctgcccagggcacagtgaggaaagaggggcagaggggatctggcatc

Exon 6

aggTgaggagaccagTtagaaagccaataagcatttccagatgagagataatggttcttgaaatccaa[→]tagtgcc
[→]caggTctaattgagatgggtgaatgaggaaaataaggaagagagaagaggcaagatggtgcctaggtttgtgat
gctcttttctgggtctcttgtctccacagGAGGAGCCATGGGGCACTACGTCTTAGCTGAACGTGAGTGAcacg
cagcctgcagactcactgtggga[←]ggagacaaaactagagactca[←]aagagggagtgcatTTatgagctcttcatg

HFE Key:

Promoter Region		Coding Regions	
Fragment 1 (HP1)	Fragment 5 (HP5)	Fragment 1 (H1)	Fragment 3B (H3B)
Fragment 2 (HP2)	Fragment 6 (HP6)	Fragment 2A (H2A)	Fragment 4 (H4)
Fragment 3 (HP3)	Fragment (HP7)	Fragment 2B (H2B)	Fragment 5 (H5)
Fragment 4 (HP4)	Fragment 8 (HP8)	Fragment 3A (H3A)	Fragment 6 (H6)
Variants: 1 = 5'UTR-1206C→G, 2 = 5'UTR-840T→G, 3 = 5'UTR-467G→C, 4 = H63D			

HAMP promoter sequence
[ENSG00000105697 (Ensembl)]

agtgccttttctgtaaagtgaaggaaatgagtgctcc[→]gacggggaggagggttctctaa[→]aaggagcagggtctgggg
agcccaggcctctgggggttgggtgactgagaaggcagcccctgaatacagagcagagctgaagggtggggcagtaa
gtgctgctgggagaacaggcagcacaggctgagttgggtgcagaagtgagtcacacatattgtccatcgtataaaaat
gtact[→]catcggactgtagatggttagc[←]tattaactattactgctatTTtatgTTTTatagacagggt[←]ctcactctgt
[→]caccaggctggagtgagtcacacaatcatagctcactgcaacctcagcctcctgggcttaagcgatctgcctc
agcctccaagtagctgggactacagatgtgtgccac[→]cacgctggctaaatttgtt[→]taaaatTTTTTTgtaga
gatgggggtctccctat[←]gttgcccaggctagcttga[→]acttctgggctcaagcgaccctcctgccttggcctcca
aattgctgggattacaggcataagccactgtgctgggcatattactgctgtcatttatggccaaaagtTTgtctc
aaacattttccagttaccagagccacatc[→]tcaagggtctgacactggg[→]aaaacaccacgtgcggatcgggacac
gctgatgcttgccctgctcagggtatctagtgttccctgccag[←]aacctatgcacgtgtggtg[→]agagcttaaagc
aatggatgcttcccccaacatgccagacactcctgaggagcctggcggctgctggccatgccccgtgtgcatgta
ggcgatgggg[→]aagtgagtgaggagagcc[→]gaaccttgattctgctcatcaaactgcttaaccgctgaagcaaaag
ggg[←]aactTTTTTccgatca[←]gcagaatgacatcgtgatgg[→]ggaaagggtcccagatggctgggtgagcagtg
tgtgtctgtgaccccgtctgccccaccccctgaacacacacctctgccgg[→]ctgagggtgacacaacct[→]gttcctg
tcgctctgttcccgttatctctcccgcctTTTtggcgccaccaccttct[←]ggaaatgagacagagcaaa[→]ggga
gggggctcagaccaccgcctcccctggcaggccccataaaagcgactgtcactcggTcccagacaccagagcaag
ctcaagaccagcagtgaggacagccagacagacggcacg[→]atggcactgagctcccagatctgggcccgttgctc
ctgctcctcctcctcctcgc[←]cagcctgaccagtggtct[→]gttttcccacaacagggtgagagcccagtggtcctggg
tccttagcagggcagcaggggatgggagagcc

HAMP coding regions

[NM_021175.2 (GenAtlas)]

Exon 1

ggctgaggggtgacacaaccctgttccctgtcgctctgttcccgttatctctcccgccttttcggcgccaccacc
 ttcttgaaatgagacagagcaaaaggggagggggctcagaccaccgcctcccctggcaggccccataaaagcgac
 tgtcactcgggtcccagacaccagagcaagcTCAAGACCCAGCAGTGGGACAGCCAGACAGACGGCACG**ATGGCAC**
TGAGCTCCCAGATCTGGGCCGCTTGCCTCCTGCTCCTCCTCCTCCTCGCCAGCCTGACCAGTGGCTCTGTTTTCC
CACAACAGgtgagagcccagtggcctgggtccttagcagggcagcagggatgggaagccaggcctcagcctagg
 gcactggagacacccgagcactgagcagagctcaggacgtctcaggagtactggcagctgaacaggaaccaggac
 aggcacgggtggctcatgcctgtaatcccagcactttgg

Exon 2

tttttttttaggaaaagccgcccattgggaaggtgagcagaagcaagaaagcaaggcccctcctaagagtccattt
 gagctctgggtttaaaccacttggagaggagcagggtgcccgggagccagtctcagaggtccactgggccccctgc
 catcctctgcaccccccttctgctttcacagACGGGACA**ACTTGCAGAGCTGCAACCCCAGGACAGAGCTGGAGCC**
AGGGCCAGCTGGATGgtgagcgcacaagctgatgcctttcctagccccctgctccctccccatgctaaggccggtt
 ccctgctcacattcccttccctccacagcccattgtccagagggcgaaggaggcgagacacccaacttccccatct
 gcattttctgctgcccgtgctgtcatcgatcaaagtgtgggatgt

Exon 3

ccatcctctgcaccccccttctgctttcacagacgggacaacttgcagagctgcaaccccaggacagagctggagc
 cagggccagctggatggtgagcgaacagtgatgcctttcctagccccctgctccctccccatgctaaggccggt
 tcctctgctcacattcccttccctcccacagCCCATGTTCCAGAGGGCGAAGGAGGCGAGACACCCACTTCCCCATC
TGCATTTTTCTGCTGCGGCTGCTGTGCATCGATCAAAGTGTGGGATGTGCTGCAAGACGTAGAACCTACCTGCCCTG
 CCCCCGTCCCCTCCCTTCCCTTATTTATTCTGCTGCCCCAGAACATAGGTCTTTGGAATAAAATGGCTGGTTCTTT
 TGTTTTCCaaaccagagtgctgtgtgctcctttctctctgcccagtgctgtgctaagagcttgtcctgacctgc

HAMP Key:

Promoter Region		Coding Regions
Fragment 1 (PP1)	Fragment 4 (PP4)	Fragment 1 (P1)
Fragment 2 (PP2)	Fragment 5 (PP5)	Fragment 2 (P2)
Fragment 3 (PP3)	Fragment 6 (PP6)	Fragment 3 (P3)
Variant: 1 = 5'UTR-335G→T		

***SLC40A1* promoter sequence**

[ENSG00000138449 (Ensembl)]

gaatccagctgcacccaccccgtagacctttggggctcctgattgagagtgcagatacagggcacatactcatgc
 gtggctcccttactactgggtgtcagtcctggcctgtgctcaaggtgtggcatctggttggagtttcaatatgta
 ggatccactaccaggggttttcgtgagattaagaagggtaaggtaacctactggcaaaaggggctggatgcccc
 ggggttggtttggcacagcaggattaaaacgaagtcaaccaaggctag¹agtcctgggtgttcttta²gtcattcacc
 tcaccctccaggaggccaccgaatggctttatctggacagggacagatccagggacacaactgggataaccggta
 ttcttggaagcctgtcactggggcatttggagggttaggtggggaagggacgcgcgcgtgggggccccgggtgagag
 ggtacaga³gggagaaggaatgatggtga⁴agggtttgctggggctgcagcatcctca⁵tctgtctccaggacggat
 ttggaggccccagtttggggatacgggtaggctctgtaactcgtgcgggacttcaccttgcagcctccgtttg
 ctctctcaagaggatggactctgatctttgcgcccccttctgcctttgattctggttctttgagg⁶gaagcc
 ctgctatgcagtc⁷ggggaaggaaaggcatttctctgctgcaggcgggcccgaatgggacggccag⁸gaaagcggcc
 tctgtggcatgaattatatttatttagata⁹ctgtattaaaaattattttcgttaaaaaaggaatccccaccac
 caagctcgcg¹⁰ctggagctttgcactcggac¹¹cgtccggcggccgcctttccct¹²aactgcggggtagccggtgc
 gcagc¹³gctttctccagcacctgac¹⁴gcttagtttgcgcagaatctccctacgcgcgcccgccggt¹⁵cacgcg
 ccttctccttttccagccccacggccgccccccgaggttgcctcgcggcttcccggagagcaggaaaaccg
 gggag¹⁶tggaacgcgtcgaggcga¹⁷gggtcccgcgaagccgcgcaggggtgtctgcggccgggttggaacgcttgcgccc
 ggggt¹⁸gggcgactcctccgggcaagggcgcggggacggcccgccgcgca¹⁹aggttgacgggagctcgtct²⁰cgcc
 cgccgggacgcccgggcccctgaaggggacggggcggccccagtcggaggtcgcagggagctccgccccgcac
 tcggtataagagctgggccccggcca²¹cgccggcggcggcggcggcgg²²agagagctggctcagggcgtccgctag
 gctcggacgacctg²³ctgagcctcccaaacgctt²⁴ccataaggctttgcctttcaa²⁵ttcagctacagtgttagc
 taagtttggaaagaaggaaaaaagaaaatccctgggccccctttcttttgttctttgccaagtctgctgtgtgag
 tctttttgccaaggctgttgtgttttagaggtgctatctccagttccttgcactcctgttaacaagcacctca
 gcgagagcagcagcagcagatagcagccgcagaagagccagcgggggtcgcctagtgt²⁶catgaccagggcgggagat
 cacaaccgc²⁷cagagaggatgctgtggtga²⁸gtgtcgttgaccgaaagcatatgggtggaaaccagggtggggctttg
 gagacaagcaactctacacgagttctggaggaatgtggctctgctgtgaaccatagctttgtaaaaagatccttt
 gactcatatttgggtggacgttaaggaagaaaggaaattcaggggtgtgggaaaaggggtttgcacacaggcacgga
 tggagtagattgggcagtttggattgccttgtgttaaaaaagaaacaaaac

SLC40A1 coding regions

[NM_014585 (GenAtlas)]

Exon 1

cggcttccccggagagcaggaaaacccgggggagtggaaacgcgctcgaggcgaaggtccccgcaagccgcgcagggtg
tctgcggccgggttgacgcttgcgcccgggggtgggcgactcctccgggcaagggcgcggggacggcccggcgcgc
aaggttgacgggagctcgtctcgcgcgcgcgGGGACGCCCGGGCGGCCCTGAAGGGGACGGGGCGGCC**CCAGTCGG**
AGGTCGCAGG→GAGCTCCGCCCCGACTCGGTATAAGAGCTGGGCCCGGCCACGGCGGCGGCGGCGGCGGAG
AGAGCTGGCTCAGGGCGTCCGCTAGGCTCGGACGACCTGCTGAGCCTCCCAAACCGCTTCCATAAGGCTTTGCCT
TTCCAACCTCAGCTACAGTGTAGCTAAGTTTGGAAAGAAGGAAAAAGAAAATCCCTGGGCCCTTTTCTTTTG
TTCTTTG**CCAAAGTCGTCGTTGTAGTC**→TTTTTGCCCAAGGCTGTTGTGTTTTTAGAGGTGCTATCT**CCAGTTCCT**
TGCACTCCTGTTAACAAGCACCTCAGCGAGAGCAGCAGCAGCGATAGCAGCCGAGAAGAGCCAGCGGGTGC
TAGTGTC**ATGACCAGGGCGGGAGATCACAACCGCCAGAGAGGATGCTGTG**gtgagtgtcgttgaccgaaagcata
tgggtgaaacccaggtggggctttggagacaagcaact**ctacacgagttctggaggaa**tgtggctctgctgtgaa
ccatagctttgtaaaaagatcctttgactcatatctgggtggacggttaaggaagaaaggaaattcaggggtggtgga
aaagg

Exon 2

aatggtattaagtgaacgaaatacatcggttcataggttaacttgataaaatgtacgtggtttgcctgcaaagta
gtttttaataatcatgttctaataagatcaaa**tgataagcattctgcctc**agctcattaagtgactaccatcg
ctttttgtcaccgccctgtgtctttgcag**GATCCTTGCCGACTACCTGACCTCTGCAAAATTCCTTCTCTACC**
TTGGTCATTCTCTCTACTTGGgtaagtgagaatgcatagctcttacaacacagttgcgcaattttttatttctt
ttcgttctagccagttgtattaagccaacttccagttttgtcaagcagttaaagaaataaat**catccaagtacac**
atgctttaatgaaaacgttatttacatcgaagatctttccccatgagtgtag

Exon 3

ccattgtgctgggatgaacggttttaacatctgagcagatattcaatctaagagtaattactgactttgaaagtctc
ataatgtagccaggaagtgccttttt**gataaggaagcaacttctctg**agtacaatagactagaaaagaaaaatatt
ccatcaaaacattttctcttttcatttaag**GGAGATCGGATGTGGCACTTTGCGGTGTCTGTGTTTCTGGTAGAG**
CTCTATGGAAACAGCCTCCTTTTGACAGCAGTCTACGGCTGGTGGTGGCAGGGTCTGTTCTGGTCTGGGAGCC
ATCATCGGTGACTGGGTGGACAAGAATGCTAGACTTAAAGgtgagtgttattatataattaagcctttttattca
tgggaccaatgcctgagctacctctgtagcaaaaggaacaaca**ctaggagagaaacaaccagg**aatgtctgc
atgccacacttgagggaggagggttagatggcaccacctctggatggagggtcccatggctcccacaca

Exon 4

tgacttcagaaagggttttctttttatctggtaataattaggtctgtgtattaatgtattatagtagaacaattat
gtgt**ggataaagaacagttctactg**agacattttgatgtaatgtacactttctctcttctctgcacag**TGGCCCA**
GACCTCGCTGGTGGTACAGAATGTTTCAGTCATCCTGTGTGGAATCATCCTGATGATGGTTTTCTTACATAAACA
TGAGCTTCTGACCATGTACCATGGATGGGTTCTCgtaagttctcaatgagattcttgatggcagaaaattgaata
tctggtagtggttaaggatgaaaatgctttgaagctatTTTTTTTTTggccagtgtagccttttaatttgatt
tctgtgtctactgtaatatcccctatagtttggtttggtt

Exon 5

cattgactactgggtattcattcagtttcataatctataacgtaaaatgatttcttataaatgaaattaaaatactt
 tttttatcattccaccaagactattttaaactgcttggtagtgacatatgtacagtgtggtaaactgacatt
 ataactcattttttcttgtcattccttag**ACTTCCTGCTATATCCTGATCATCACTATTGCAAATATTGCAAAT**
TTGGCCAGTACTGCTACTGCAATCACAATCCAAAGGGATTGGATTGTTGTTGTTGCAGGAGAAGACAGAAGCAA
CTAGCAAgtaatttggctttctcttttaaatgaaatgagcatgtaggattcactttaaat**cggtggtgataaatg**
aggctgtaagccttgtatttttgggtattttttaagaatgataaattgaaagcatactttttttcttacc
 ttattgtcagtttttagtgctgatttatctcactgtta

Exon 6

gtgggacttgaccacaacaacaatatttttccaacaaaatgtctttcttacaatgtacttttagaaaaccaca
 ttttaggaatctatactcttggtttacagcttggtagt**ttgtgtaaatgggcagtct**cttttgatgggttgcaca
 cttacctgcctctttcacctgcctctctag**ATATGAATGCCACAATACGAAGGATTGACCAGTTAACCAACATCT**
TAGCCCCATGGCTGTTGGCCAGATTATGACATTTGGCTCCCCAGTCATCGGCTGTGGCTTTATTTCCGGATGGA
ACTTGGTATCCATGTGCGTGGAGTACGTT⁶**CTGCTCTGGAAGGTTTACCAGAAAACCCAGCTCTAGCTGTGAAA**
GCTGGTCTTAAAGAAGAGGAAACTGAATTGAAAACAGCTGAATTTACACAAAAGgtaaaactgaacacaatgatctct
 ccttttggtt**ctcatgctcagaccttaaatg**ttggtagaatcaaaactattttgaatttgatcaggttttatta
 ccagtgggggcccagatgaggttaaataatctgctttggtagacgaggaagagcaggcttttgaggatctagga
 aaaactc

Exon 7

acttgatgattattccttggctggaattccttagattattagtaaaagaaaatacatattacaatgtctaaccaag
 ggtaccattgggaaggggaatagaaggaaaaaaagtactactaataat**gcttttatttctacatgtcc**ctcc
 caacaaaataatgggtatcttttcttaacag**ATACTGAGCCAAAACCCCTGGAGGGAACTCATCTAATGGGTGTGA**
AAGACTCTAACATCCATGAGCTTGAACATGAGCAAGAGCCTACTTGTGCCTCCCAGATGGCTGAGCCCTTCCGTA
CCTTCCGAGATGGATGGGTCTCCTACTACAACCAGCCTGTGTTTCTGGCTGGCATGGGTCTTGCTTTCTTTATA
TGACTGTCCTGGGCTTTGACTGCATCACCACA**GGGTACGCCTACACTCAG**GGACTGAGTGGTTCCATCCTCAGTA
 TTTTGATGG**GAGCATCAGCTATAACTGGA**ATAATGGGAAGTGTAGCTTTTACTTGGCTACGCGAAAATGTGGTT
 TGGTTCCGACAGGTCTGATCTCAGGATTGGCACAGCTTTTCTGTTTGTGATCTTGTGTGTGATCTCTGTATTCATGC
 CTGGAAGCCCCCTGGACTTGTCCGTTTCTCCTTTT**GAAGATATCCGATCAAGGTT**CATTCAAGGAGAGTCAATTA
 CACCTACCAAG**GATACTGAAATTACA**ACTGAAATATACATGTCTAATGGGTCTAATTCTGCTAATATTGTCCCGG
 AGACAAGTCTGAATCTGTGCCATAATCTCTGTGAGTCTGCTGTTTGCAGGCGTCATTGCTGCTAGAATCGgta
 agaaatctctttttatataatgaactaaagtgtcttttggtaat**gtaggttcagagaaatccatta**aaatg
 atctgaaatgtccctaaatgtaatttaagcaaaatccactcttacgaaatttttattttacatatttatactt
 tatattttattgtgttttttattttata

Exon 8

ataaccaatattttatgaaaaataattcttaaggcaaggctatgggtatatttaagggtgacttaagacagt
caggctaaaatgtatattttgcatatgtcaacagatttttatctgtgat**ttgaaatgtatgctgtaaac**taaaa
tctaactctttaaaaaaatattttattatag**GTCTTTGGTCCTTTGATT**TAACTGTGACACAGTTGCTGCAAGAA
ATGTAATTGAATCTGAAAGAGGCATTATAAATGGTGTACAGAACTCCATGAACTATCTTCTTGATCTTCTGCATT
TCATCATGGTCATCCTGGCTCCAAATCCTGAAGCTTTTGGCTTGCTCGTATTGATTTCAGTCTCCTTTGTGGCAA
TGGGCCACATTATGTATTT**CGATTTGCCAAAATACT****CTGGGAAACAAGCTCTTTGCTTGCGGTCCTGATGCAA**
AAGAAGTTAGGAAGGAAAATCAAGCAAATACATCTGTTGTTTGAGACAGTTTAACTGTTGCTATCCTGTTACTAG
ATTATATAGAGCACATGTGCTTATTTTGTACTGCAGAATTCCAATAAATGGCTGGGTGTTTGTCTCT**GTTTTTAC**
CACAGCTGTGCCTTGAGAACTAAAAGCTGTTTAGGAAACCTAAGTCAGCAGAAATTAAGTATTAATTT**CCCTTA**
TGTTGAGGCATGGAAAAAAAAATTGGAAAAGAAAAACTCAGTTTAAATACGGAGACTATAATGATAACACTGAATT
CCCCTATTTCTCATGAGTAGATAACAATCTTACGTAAAAGAGTGGTTAGTCACGTGAATTCAGTTATCATTTGACA
GATTCTTATCTGTACTAGAATTCAGATATGTCAGTTTTCTGCAAAACTCACTCTTGTTCAGACTAGCTAATTTA
TTTTTTTGCATCTTAGTTATTTTTAAAAA**CAAATTTCTCAAGTATGAAGAC**TAAATTTTGATAACTAATATTATC

SLC40A1 Key:

Promoter Region		Coding Regions	
Fragment 1 (SP1)	Fragment 5 (SP5)	Fragment 1A (S1A)	Fragment 7A (S7A)
Fragment 2 (SP2)	Fragment 6 (SP6)	Fragment 1B (S1B)	Fragment 7B (S7B)
Fragment 3 (SP3)	Fragment 7 (SP7)	Fragment 2 (S2)	Fragment 7C (S7C)
Fragment 4 (SP4)		Fragment 3 (S3)	Fragment 8A (S8A)
		Fragment 4 (S4)	Fragment 8B (S8B)
		Fragment 5 (S5)	Fragment 8C (S8C)
		Fragment 6 (S6)	
Variants: 1 = 5'UTR-1355G→C, 2 = 5'UTR-750G→A, 3 = 5'UTR-593C→T, 4 = 5'UTR-501T→C, 5 = (CGG) repeat, 6 = V221			

***CYBRD1* promoter sequence**

[ENSG00000071967 (Ensembl)]

ggggctttgggttcaaattgggtttgggtggggcgggcactttgaaaatccatttgtcacactaaacggcaagtccag
 gtccaggagggttctctgtcttccctctctcaagagcaaaactgcaagtagtttcattgcaggataaggccaagccca
 ccgctgccccatgctgtttttgttttccagacagagcttagctgtcaccaggctgcagtgcagtggtgcgat
 cat¹ac¹ctctcctgag²ctcaagtgattctcttgctc³cacctcccaaagccctgggattacagctgtgagacac
 ctcatggggac⁴ccggtttactgggtttttttgtttttgtttttgttttttt⁴gagacagagctctcacttt
 gtcacccaagctgaagtgcagtggtgcaacctcagctcactgcagcctcgaccttctgggctcaagtgatcctcc
 ttcc⁵tcagtcccccaagtagctggggctacaggtgcatgcatttgtattt⁵cagtagagacagggtttca⁵cttg
 ttgccaggctgttctcaaactcctggactcaagtgatctgccgccttagcctcccaaagtgctaagattacag
 gtgtgaggca⁶ctgcgcct⁶ggcctt⁶actgt⁶taacttaaacaacaaaattataaattgaaaaagaggagactt
 ttttcttataaaaag⁷gttatagcctgcaaggagg⁷cattccataggctgataaacatagcctctggcctaagacc
 agagacaggcacttggaaaggcagaggggttggggtaggagctttatgctgaacagt⁷ttggccaacatacatac
 gtaac⁸aggttacaggaggagctat⁸aatattaatggagggtggtccttacacatgcatattgaacaaa⁸catgcatg
 taacatg⁹ttctctttgggggtggagacttaacatttaattgtattacttcaaatacat⁹ttaaatgtattacttcaa
 acctacacttcaaaaggctctttcaggacgtgaatgcatacaagtgcacaatcc⁹ctgtacactggccagaaccg⁹
 tccatggctcggctcttcttatcatgaaaaagttcctgaaatcagcccagtgaaag¹⁰ctgtagttctggctggtgca¹⁰
 caggggttcagctggctcagcatctgtgaactgattaagtgtgaattgttttaataattgcttatctcaagccagtg
 ctgttttagcctctagaggaaaagaaaacctt¹⁰gtggcagttagaccatagtttatttcttaagt¹⁰gtaggagtg¹⁰
 gtgacttagg¹¹tcctttttataat¹¹ttgatgtcttattgctacaa¹¹agagctctgttctgtccgcct¹¹tatgat¹¹ttctat
 tttaaacattaatgctagtcagctgttgagtctaaattccaaaatggagggggtagacttcccttccggctgtag
 ctagaaactcagctttaagggtttttctgg¹²gtctgcttggccaaggag¹²ggtccattcagtcagtgagg¹²ggcttag
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 cttaaccaagagggcgccactgcctactcaacctccccacaaata¹⁴aggacgagacacgggaagt¹⁴cttaaag
 caagt¹⁵ggtgagtataaaatcctcggaat¹⁵tttctctttgagcaactaactaggctctgttactg¹⁵aaagcctctgca
 gctt¹⁶gatgctggacgagggagaggggtgaggccaccaggca¹⁶tgagcgccctcggcgggcgcgctgatcccgggg
 gtggggcc¹⁷catttctgagttggggccag¹⁷ctccccacc¹⁸caag¹⁸aggccccacattccgggcccagcagcccag
 aaagtcctccccgcagggcggagacagccccagaagtcgacgccccgggtcccgccgcccggccactaccagag
 ggctgccgcccctctccaagtcttgtggcccccggggtgaggatgagggcgctgatggggcagtgatgggggct
 ctggcgcttctggcgctgctggggctggcactgctcgtcggc¹⁹ctctgtcgggtgatcttgc¹⁹cctcgtctgggt
 cctccactaccgagagggggcttggctgggatgggagcgcactagagtttaactggcaccacagtgctcatggtcac
 cggcttctgtcttcatccagggcatcggtaactggcacc

Exon 4

tcaatttcattatatattccccgagtggaattgtagcataatttatatttctccttaatagcaagctttgagagttt
 acattgaaactgtactagtgtgcatTTTTgagcagataaattcaagactgttttgcattggtgctgtatcatcctgt
 ttgtaattggatacatctcttatttcatagGAGAGATCCTGCATACAGTACATTCCCGCCAGAAGGTGTTTTCGT
 AAATACGCTTGGCCTTCTGATCCTGGTGTTCGGGGCCCTCATTTTTTTGGATAGTCACCAGACCCGAATGGAAACG
 TCCTAAGGAGCCAAATTCTACCATTCTTCATCCAAATGGAGGCACTGAAACAGGAGCAAGAGTTCCATGCCAGC
 CTACTCTGGCAACAACATGGACAAATCAGATTGAGAGTTAAACAG¹³TGAAGTAGCAGCAAGGAAAAGAACTTAG
 CTCTGGATGAGGCTGGGCAGAGATCTACCATGTAAaatggtgtagagatagagccatataacgtcacgtttcaaa
 actagctctacagtttttgcttctcctatttagccatattgataattgggctatgtagtatcaatatttactttaatc
 acaaaggatgggttcttgaataaattgtattgattgaggcctatgaactgacctgaattggaaaggatgtgatt

CYBRD1 Key:

Promoter Region		Coding Regions	
Fragment 1 (BP1)	Fragment 6 (BP6)	Fragment 1 (Cy1)	Fragment 4A (Cy4A)
Fragment 2 (BP2)	Fragment 7 (BP7)	Fragment 2 (Cy2)	Fragment 4B (Cy4B)
Fragment 3 (BP3)	Fragment 8 (BP8)	Fragment 3 (Cy3)	
Fragment 4 (BP4)	Fragment 9 (BP9)		
Fragment 5 (BP5)	Fragment 10 (BP10)		
<p>Variants: 1 = 5'UTR-1844C→G, 2 = 5'UTR-1834G→A, 3 = 5'UTR-1813C→T, 4 = G(T)₈G(T)₆G(T)_nG(T)₉ repeat, 5 = 5'UTR-1459T→C, 6 = 5'UTR-1452T→C, 7 = 5'UTR-1272T→C, 8 = 5'UTR-624G→A, 9 = 5'UTR-238A→G, 10 = 5'UTR-167C→G, 11 = 5'UTR-163G→A, 12 = IVS2+8C→T, 13 = S266N</p>			

***HJV* promoter sequence**
[ENS00000168509 (Ensembl)]

agaagagtgctatgagggcctctagactctgtattaaaatagagccaactggtaaaga **tggttagtgattgtgt**
tggttattactgagtgtcaatttgattggattgaaggatacaaagtattgatcctgggtgtgtctgtgaggggtg
 tgccaaaagaaattaacatttgagtcagtgggctgggaaaggcagatccacccttaatctgggtgagcacaatct
 aattcactgccagcacagctagaataaaaagcaggcagaaaaatatgaaaggagagactggc¹tagcctcccag
 ccta **catatttctcccatgctgga**tgcttcctgcccttgaacatcagactccaagttcttcaattttgaga**ctga**
gactggctctccttgcccctcaagcttgcagacagcctactgtgggacctgtgatcgtgtaagttaacttaa
 taaattccccttttatttatatctacctatatagatatccatatctatatagatattaataaatctagagagac
 a**gaaagcagactgggtgatggc**cagtctagatggctagatagatagacatggatatagatatagatctctatatag
 atagaggtagatacagatatagatatatgccctattagttctgttcctctagagaaccctaatacag**tgaccgta**
gaccgta
tttgaatcggtcctt
tttgaatcggc
 ctgtaatttctacttggcaagtactaaaagatgatgatctcagatataacctatggctgcaaaaacatgacat**ggc**
taaatcccttgggttgcagtatctcttttcttttttaaggggggtgggggggagggtctcactgttgcccaggctg
 gagtgcaatggcgttat **catagctcactgcagcctc**aaactcctgcgctcaagtgaccctcctgc**ctcagctcc**
aaagtgctgagattttgcaatatttatggtcacaagattatggtattccataaaagtatctttctgaggctaggc
 atggttggtcacacttghtaatcccagcactctgagaggctgag**gatggaaggattcattgaggc**aaggagttcaag
 accagcctgggtcaacatagtgaga**ctcctcatctcggaaggaagga**aggaaggaggaggaggga²agggaggaggt
 gaaggaaggaaggaaggaagg⁴aag⁵gaaggaaggaaggaaggaaggaagg³aaaagtatattttgaaatct
 ttttctatttctccaactctttctttagaagaattctatttccattctttcttccactctttgcctttgttag**cc**
ttctctccaagcaaatcgggagcctttatttttgggtattcatgag**ggagaggaagatgaattgctg**tacaac
 taaagtaatgaaaatggagtaggtaggagatagacagctgcaaggatctgagctggatagactgaacaaacct
 catcctaagcaactcacagctcagattt **cttctctggacagctggct**tttttctgccttctgaaatactctgcaa
 agataggagaggggcta**tgaactacctctgctatgg**atcttattcaaagtcagctacctcctagatactatctgt
 agaacctaaatgtaatat **tagcatagcagggatgaac**atggtaaatgaaaggatccaattgccactgtaatt
 tttaaaggccaggagctcaacattattgaaa**atg**ctggagggt**ccctggagtaggcagtgac**cacagagtcaca
 caagctggaattggatatccaacttgtctgtcatatttctctcctccctccctgacttggcactcaatactccat
 attct**ttctaactctctaaccctcc**ccactcccccaactcccacacctacccccaccaa**cgttcctggaatttt**
ggaacttagctatttttaaaaccgtcaactcagtagccacctccctccctgctcagctgtccagtaactctggccag
 ccataactcccccttccccccataccaaaccttctctgggtccctgacctcagtgagacagcagccggcctggg
 gacctgggggagacacggaggaccccctggctggagctgacctacagagtagggaatcatggctggagaattgga
 tagcagagtaatgttt**gacctctggaacagtaagt**caaaatgaaattgcaattcctttaa

HJV coding regions

[ENS000001698509 (Ensembl)]

Exon 1

taaaggtatccaattgcccactgtaatttttaaagccaggagctcaacattattgaaa**ATGCTGGAGGGCTGCCTGGAGT**
AGGCAGTGACCACAGAGTACACAAGCTGGAATTGGATATCCAACCTTGTCTGTTCATATTTCTCTCCCTCCCTCCCT
GACTTGGCACTCAATACTCCATATTCTTTCTAATCTCTAACCCCTCCCCACTCCCCAACTCCCACACCCCTACCC
CCACCAACGTTCTGGAATTTTGGACTTAGCTATTTTTAAAACCGTCAACTCAGTAGCCACCTCCCTCCCTGCTC
AGCTGTCCAGTACTCTGGCCAGCCATATACTCCCCCTTCCCCCATAACCAAACCTTCTCTGGTTCCCTGACCTCA
GTGAGACAGCAGCCGGCCTGGGGACCTGGGGGAGACACGGAGGACCCCTGGCTGGAGCTGACCCACAGAGTAGG
GAATCATGGCTGGAGAATTGGATAGCAGAGTAATGTTTGACCTCTGGAAACAgtaagtcaaaatgaaattgcaat
 tcctttaataagcttttatattgaagttagacttttataaaattacaaacacctacttggatg**tctctcgtccaa**
atgctgggatctctccctaccaaggtgccccaatctccatttctctttctgtcttatttctttctggcctctggc
 ctctagctttttgaagtttaattctctgtctctcctctggcagtccttagccctctctttaccttattacctcaag

Exon 2

ggcttaactgccacacttatagtttgaggaactccaatctccccaaattccagtctgttcatccttttcttgatc
 tccccagatt**cactccacattatcctta**caatcttcaattcttctctctctccatgtccagccaaatttctttt
 ttcag**TCACCTTACAGGGCTTCCGGTCAAAATTC**ACTAGGTAGGAGGGTCATCAGCTGGGAAGAACC GGCGCCTGG
GAAACCTGGCTGGATAGGTATGGGGGAGCCAGGCCAGTCCCCTAGTCCCAGGTCCTCCCATGGCAGTCCCCAAC
TCTAAGCACTCTCACTCTCCTGCTGCTCCTCTGTGGACATGgtaaggaagggccaggggaaggggttggggaaatc
 tagagggtaggct**gctatgtaggggtgggcat**gtgagcctgaatgagtgaggagagataggcgctgagagtcccg

Exon 3

agatgtggcaggcttacacacttttagtaagacagccgagagaactagggactaggggggttgggggctgggggaa
 gcccttagttaggttttaggaaggtggaaccctgatgagatttggagagttatgagcaaaact**acactccga**
tagagcagaggtctgaggaccgtctcacaatcctctcctctctgtcttttag**CTCATTCTCAATGCAAGATCTCC**
GCTGCAATGCTGAGTACGTATCGTCCACTCTGAGCCTTAGAGGTGGGGGTTTCATCAGGAGCACTTCGAGGAGGAG
GAGGAGGAGGCCGGGGTGGAGGGGTGGGCTCTGGCGGCCTCTGTGCGAGCCCTCCGCTCCTATGCGCTCTGCACTC
GGCGCACCGCCCGCACCTGCCGCGGGACCTCGCCTTCC
GACCTGATGATCCAGCACAA
ATTCCGGCGGTACATGGCATCGAAGACCTGATGATCCAGCACAACTGCTCCCGCCAGGGCCCTACAGCCC
 CTCCCCGCCCCGGGGCCCCGCCCTTCCAGGCGGGGCTCCGGCCTCCCTGCCCGGAC**CCTTGTGACT**
CCGACCTTGTGACT
ATGAAGGCCGGTTTTCCCGCTGCATGGTCGTCCCCGGGGTTCTTGCATTGCGCTTCTTCCGGGGACC
ATGA
 CCCATGTGCGCAGCTTCCACCATCACTTTACACATGCCGTGTCCAAGGAGCTTGGCCTCTACTGGATAAT**GACT**
TCCTCTTTGTCCAAGCCACCAGCTCCCCATGGCGTTGGGGGCCAACGCTACCGCCACCCGGAAGgtcaggcact
 caatcttcttccgatccacctcat**gagattcttccacgggac**cattcctccccatccccactattcaacagca

Exon 4

t t t c c c t c t c c t a g g a a g t t g c c a c g a t t a a g t a g a g a g g g g g t t a a g t a g g g a t g a g g t a a t a c t g g a a c a t a a
a t a g g a g a a g g g a t c a a g g a t t g a g g g c c a t a g t a g t c t c t g c a t c t c t a c t t g g a t c a g a t c t c t a a c t a t g t a t
g a g g t c t g a t t g g g g g a a g a t g c a c t g a a c c c a a a a t g a a c t g t t t t c c c t c t t g t c c t c a c a g C T C A C C A T C A
T A T T T A A G A A C A T G C A G G A A T G C A T T G A T C A G A A G G T G T A T C A G G C T G A G G T G G A T A A T C T T C C T G T A G C C T T T G
A A G A T G G T T C T A T C A A T G G A G G T G A C C G A C C T G G G G G A T C C A G T T T G T C G A T T C A A A C T G C T A A C C C T G G G A A C C
A T G T G G A G A T C C A A G C T G C C T A C A T T G G C A C A A C T A T A A T C A T T C G G C A G A C A G C T G G G C A G C T C C T T C T C C A
T C A A G G T A G C A G A G G A T G T G G C C A T G G C C T T C T C A G C T G A A C A G G A C C T G C A G C T C T G T G T T G G G G G T G C C C T C
C A A G T C A G C G A C T C T C T C G A T C A G A G C G C A A T C G T C G G G A G C T A T A A C C A T T G A T A C T G C C A G A C G G C T G T G C A
A G G A A G G G C T T C C A G T G G A A G A T G C T T A C T T C C A T T C C T G T G T C T T T G A T G T T T T A A T T T C T G G T G A T C C C A A C T
T T A C C G T G G C A G C T C A G G C A G C A C T G G A G G A T G C C C G A G C C T T C C T G C C A G A C T T A G A G A A G C T G C A T C T C T T C C
C C T C A G A T G C T G G G G T T C C T C T T T C C T C A G C A A C C C T T A G C T C C A C T C C T T T C T G G G C T C T T T G T T C T G T G C
T T T G C A T T C A G T A A G G G A C C A T C A G T C C A T T A C T A G T T T G G A A A T G A T T T G G A G A T A C A G A T T G G C A T A G A A G
A A T G T A A G A A T C A T T A A A G G A A G C A G G G C C T A G G A G A C A C G T G A A A C A A T G A C A T T A T C C A G A G T

C A G A T G A G G C T G C A G T C C A G G G T T G A A A T T A T C A C A G A A T A A G G A T T C T G G G C A A G G T T A C T G C A T T C C
G G A T C T C T G T G
G G A T C T C T G T G G G G C T C T T C A C C A A T T T T T C A G C C T C A T T T A T A G T A A A C A A A T T G T T C T A A T C C A T T T A C T G C
A G A T T T C A C C C T T A T A A G T T T A G A G G T C A T G A A G G T T T T A A T G A T C A G T A A A G A T T T A A G G G T T G A G A T T T T T A A
G A G G C A A G A G C T G A A A G C A G A A G A C A T G A T C A T T A G C C A T A A G A A A C T C A A A G G A G G

T G T G T G T A A G G T A T G T T C T G C
A A G A C A T A A T T A G G G A A A G A A G T C T A T T T G A T G A A T A T G T G T G T G T A A G G T A T G T T C T G C T T T C T T G A T T C A A A A
A T G A A G C A G G C A T T G T C T A G C T C T T A G G T G A A G G G A G T C T C T G C T T T T G A A G A A T G G C A C A G G T A G G A C A G A A G T
A T C A T C C C T A C C C C T A A C T A A T C T G T T A T T A A A G C T A C A A A T T C T T C A C A C C A T C c t c t g t t g c c t a t g t t g a a
t c t c t t t a c a g a t g c t t g a a a t g g a g t a a a t g c a a t g t g t t c a c t c c a c t g a a g a g g g c t c g g a a g t a t c a g a t
a c t g t t g c t a t c t c a g g g a g t t t a c a g g c t a t t g g a g a g a c a a a c c a a t t c a c a t g a a a g a g t g a t g a g t g t g t

HJV Key:

Promoter Region		Coding Regions	
Fragment 1 (JP1)	Fragment 6 (JP6)	Fragment 1 (J1)	Fragment 4B (J4B)
Fragment 2 (JP2)	Fragment 7 (JP7)	Fragment 2 (J2)	Fragment 4C (J4C)
Fragment 3 (JP3)	Fragment 8 (JP8)	Fragment 3A (J3A)	Fragment 4D (J4D)
Fragment 4 (JP4)	Fragment 9 (JP9)	Fragment 3B (J3B)	Fragment 4E (J4E)
Fragment 5 (JP5)	Fragment 10 (JP10)	Fragment 3C (J3C)	Fragment 4F (J4F)
		Fragment 3D (J3D)	Fragment 4 G (J4G)
		Fragment 4A (J4A)	Fragment 4H (J4H)

Variants: 1 = 5'UTR-1406C→A, 2 = 5'UTR-542A→G, 3 = (AAGG) repeat,
4 = 5'UTR-534G→T, 5 = 5'UTR-530G→T

APPENDIX 3: GENOTYPES OF TWO INDIAN PROBANDS AND EXTENDED FAMILY MEMBERS.

The genotypes of the two Indian probands and extended family members are provided in the tables below. The genotypes are denoted WT when the individual presented with the homozygous wild type genotype, HET when they were heterozygous and HOM when they presented with the homozygous variant genotype. The genotypes of some of the individuals are not indicated. Genotypes were omitted for three possible reasons: either 1) amplification of the relevant DNA fragment was unsuccessful and mutation analysis was incomplete, 2) DNA fragments were successfully amplified and HEX-SSCP analysis was performed but the genotype of the individual was unclear or 3) results from HEX-SSCP, RFLP or semi-automated DNA sequencing analysis were ambiguous and genotypes were not consistent with the haplotypes present in the family. The genotypes of these individuals will be clarified employing semi-automated DNA sequencing analysis. The reason for the ambiguity (option 1, 2 or 3) is colour coded and the key is given at the end of this appendix.

HMOX1 gene

Sample	Variants		
	5'UTR-495A/T	IVS2+4T/C	IVS2 -19C/T
Proband 1	HET	HET	WT
Proband 2	HET	HET	HET
II:2	WT	HET	WT
II:4	HET	WT	WT
II:5	HET	WT	WT
II:6	HET	WT	WT
II:7	HET	WT	WT
III:1	HOM	WT	WT
III:2	-	WT	WT
III:3	HOM	WT	WT
III:4	WT	WT	WT
III:5	WT	WT	WT
III:7	-	HET	HET
III:8	HET	HET	HET
III:9	HET	-	WT
III:10	HET	HET	HET
III:12	-	HET	HET
III:14	HOM	WT	WT
III:16	HOM	WT	WT
III:17	HOM	WT	WT
III:19	HOM	WT	WT
III:20	HOM	WT	WT
III:21	HOM	WT	WT
III:22	WT	WT	WT
III:23	WT	WT	WT

HFE gene

Sample	Variants			
	5'UTR-1206C/G	5'UTR-840T/G	5'UTR-467G/C	H63D
Proband 1	HET	HET	HET	WT
Proband 2	HET	HET	HET	WT
II:2	WT	HET	WT	-
II:4	WT	HET	WT	WT
II:5	HET	WT	HET	WT
II:6	HOM	WT	HOM	-
II:7	HET	WT	HET	WT
III:1	HET	WT	HET	HET
III:2	HET	WT	HET	HET
III:3	WT	-	WT	WT
III:4	WT	HET	WT	WT
III:5	WT	WT	WT	WT
III:7	HET	WT	HET	WT
III:8	HET	WT	HET	WT
III:9	HET	WT	HET	WT
III:10	WT	-	WT	WT
III:12	HET	-	WT	WT
III:14	WT	WT	WT	WT
III:16	WT	WT	WT	WT
III:17	WT	WT	WT	WT
III:19	HOM	WT	HOM	HET
III:20	HET	WT	HET	WT
III:21	WT	-	WT	HET
III:22	WT	WT	WT	WT
III:23	WT	WT	WT	WT

HAMP gene

Sample	Variant
	5'UTR-335G/T
Proband 1	HOM
Proband 2	HOM
II:2	HET
II:4	HET
II:5	HET
II:6	HET
II:7	WT
III:1	HET
III:2	HET
III:3	HET
III:4	WT
III:5	HET
III:7	HET
III:8	HET
III:9	HET
III:10	HET
III:12	HET
III:14	WT
III:16	WT
III:17	HET
III:19	WT
III:20	HET
III:21	WT
III:22	WT
III:23	HET

SLC40A1 gene

Sample	Variants					
	5'UTR-1355G/C	5'UTR-750G/A	5'UTR-593 C/T	5'UTR-501 T/C	(CGG) repeat *	V221V
Proband 1	HET	HOM	HOM	HOM	8	HET
Proband 2	HET	HET	HOM	HOM	8	WT
II:2	HET	HOM	HOM	HOM	8	HET
II:4	HOM	HET	HOM	HOM	8	HET
II:5	HET	HET	HOM	HOM	8	WT
II:6	HET	HOM	HOM	HOM	8	HET
II:7	HOM	HET	HOM	HOM	8	HET
III:1	HOM	HET	HET	HOM	7/8	HET
III:2	WT	HET	-	-	7/8	HET
III:3	HET	HET	HET	HOM	7/8	HET
III:4	HOM	HOM	HOM	HOM	8	HET
III:5	HET	HOM	HOM	HOM	8	HET
III:7	HET	HET	HET	HOM	7/8	WT
III:8	HOM	HET	HOM	HOM	7/8	HET
III:9	HET	WT	HET	HOM	7/8	HET
III:10	HET	HET	HET	HOM	7/8	WT
III:12	HOM	HET	HET	HOM	7/8	HET
III:14	HET	HET	HOM	HOM	8	WT
III:16	HOM	HET	HOM	HOM	8	HET
III:17	HET	HET	HOM	HOM	8	HET
III:19	HET	HOM	HET	HOM	7/8	WT
III:20	-	-	HOM	HOM	8	WT
III:21	HET	WT	HET	HOM	7/8	HET
III:22	HET	WT	HET	HOM	7/8	HET
III:23	HOM	HET	HOM	HOM	7/8	WT

* The genotype of each individual is indicated with the number of (CGG) trinucleotide repeats presented.

CYBRDI gene

Sample	Variants							
	5'UTR-1844C/G	5'UTR-1834G/A	5'UTR-1813C/T	G(T) ₈ G(T) ₆ G(T) _n G(T) ₉	5'UTR-1459T/C	5'UTR-1452T/C	5'UTR-1272 T/C	
Proband 1	HOM	HET	WT	HET*	HET	HET	HET	
Proband 2	HOM	HET	WT	HET*	HET	HET	HET	
II:2	HOM	HET	WT	HET*	HET	HET	HET	
II:4	HET	HET	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	WT	
II:5	HOM	HET	WT	HET*	HET	-	WT	
II:6	HET	HET	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	-	
II:7	HET	HET	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	WT	
III:1	-	-	-	-	-	-	WT	
III:2	HOM	WT	WT	G(T) ₈ G(T) ₆ G(T) ₉	HOM	WT	WT	
III:3	HOM	HET	WT	HET*	HET	HET	WT	
III:4	HOM	HOM	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	HET	
III:5	HOM	HOM	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	-	
III:7	HOM	WT	HET	G(T) ₈ G(T) ₆ G(T) ₉	HOM	HET	HET	
III:8	HOM	WT	HET	G(T) ₈ G(T) ₆ G(T) ₉	HOM	-	WT	
III:9	HOM	HET	HET	HET*	HET	-	HET	
III:10	HOM	WT	HET	G(T) ₈ G(T) ₆ G(T) ₉	HOM	HET	WT	
III:12	HOM	HOM	WT	G(T) ₈ G(T) ₆ G(T) ₆ G(T) ₉	WT	HET	-	
III:14	HOM	HET	WT	HET*	HET	HET	WT	
III:16	HOM	HET	WT	HET*	WT	HET	WT	
III:17	HOM	HET	WT	HET*	HET	HET	WT	
III:19	HOM	HOM	WT	G(T) ₈ G(T) ₆ G(T) ₉	WT	WT	WT	
III:20	HET	-	HET	HET*	HET	HET	HET	
III:21	HOM	HET	WT	HET*	HET	WT	WT	
III:22	HOM	HET	WT	HET*	HET	HET	WT	
III:23	HET	WT	WT	HET*	HET	HET	WT	

*HET indicated individuals who presented with allele 1 (G(T)₈G(T)₆G(T)₆G(T)₉) and allele 2 (G(T)₈G(T)₆G(T)₉) of the repeat.

CYBRD1 gene (Cont.)

Sample	Variants				
	5'UTR-624G/A	5'UTR-238A/G	5'UTR-167C/G	5'UTR-163G/A	S266N
Proband 1	HET	HET	HET	HET	HET
Proband 2	HET	HET	HET	HET	HET
II:2	HET	HET	HET	HET	HET
II:4	HET	WT	WT	WT	HET
II:5	HET	HET	HET	HET	HET
II:6	HET	WT	WT	WT	HET
II:7	HET	WT	WT	WT	HET
III:1	HET	HET	HET	HET	WT
III:2	WT	HOM	HOM	HOM	HET
III:3	HET	HET	HET	HET	WT
III:4	-	WT	WT	WT	-
III:5	HET	-	WT	WT	-
III:7	-	HOM	HOM	HET	-
III:8	-	HOM	HOM	HET	-
III:9	HET	HET	HET	WT	HET
III:10	-	HET	HOM	HET	-
III:12	HOM	WT	WT	WT	-
III:14	HET	HET	HET	HET	HET
III:16	HET	WT	WT	WT	HET
III:17	HET	HET	HET	HET	HET
III:19	-	WT	WT	WT	-
III:20	WT	HET	HET	WT	HOM
III:21	HET	HET	HET	HET	HET
III:22	HET	HET	HET	HET	HET
III:23	HET	HET	-	-	HOM

HJV gene

Sample	Variants				
	5'UTR-1406C/A	5'UTR-542A/G	(AAGG) repeat *	5'UTR-534G/T	5'UTR-530G/T
Proband 1	HET	HET	12	HET	HET
Proband 2	HET	HET	12	HET	HET
II:2	HET	HET	11	HET	WT
II:4	HET	HET	11/12	HET	WT
II:5	HET	HET	12	HET	WT
II:6	HET	-	11/12	HET	HET
II:7	HET	HET	12	-	HET
III:1	HET	WT	12	WT	-
III:2	HET	WT	12	WT	HET
III:3	-	HOM	11/12	HET	HET
III:4	HOM	HOM	11/12	HET	HET
III:5	HOM	-	-	-	-
III:7	HET	HOM	12/13	HET	HET
III:8	HET	HOM	12/13	HET	HET
III:9	WT	HET	12/13	HET	HET
III:10	WT	HET	12/13	HET	HET
III:12	HET	HOM	12/13	HET	HET
III:14	HET	WT	11	WT	WT
III:16	HET	WT	11	HET	WT
III:17	WT	-	12	WT	HET
III:19	HET	HET	11/12	WT	HET
III:20	-	-	11	HET	WT
III:21	HET	HET	11/12	-	WT
III:22	HET	HET	11/12	-	WT
III:23	HET	WT	12	HET	HET

* The genotype of each individual is indicated with the number of (AAGG) tetranucleotide repeats presented.

Key to reason for omission of genotypes:

- (1) Unsuccessful amplification
- (2) HEX-SSCP analysis was performed but the results were unclear
- (3) Inconsistent genotype

APPENDIX 4: ABSTRACT OF WORK PRESENTED AT 2006 SASHG CONGRESS.

Oral presentation: 12th biennial Congress of the South African Society of Human Genetics (SASHG) congress, Golden Gate – March 2007.

IRONING OUT HAEMOCHROMATOSIS: A STUDY OF AN INDIAN FAMILY

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Hereditary haemochromatosis (HH) is a disease characterised by iron overload. Excess iron can cause damage to various tissues and organs if the disease goes untreated. Most cases of HH in Caucasians of European descent are caused by mutations in the haemochromatosis (*HFE*) gene. Various other genes have also been found to play a role in other forms of haemochromatosis. The disease demonstrates genetic heterogeneity. This heterogeneity has hinted at the possibility of modifier genes altering the disease phenotype. In this study an Indian family with classic clinical symptoms of *HFE* haemochromatosis, but no *HFE* mutations, was investigated. The two probands, a brother and sister, both have typical hereditary haemochromatosis but do not have the common C282Y or H63D mutations in the *HFE* gene. The probands also do not have any of the less common mutations in the *HFE* gene. Seven genes involved in iron metabolism were screened (promoter and coding region). These genes are the genes that encode cytochrome b reductase 1 (*CYBRD1*), heme oxygenase 1 (*HMOX1*), hepcidin antimicrobial peptide (*HAMP*), hemojuvelin (*HJV*), ferroportin 1 (*SLC40A1*), ceruloplasmin (*CP*), and the high iron gene *HFE*. Mutation analysis of these genes was performed using polymerase chain reaction (PCR), heteroduplex single strand conformational polymorphism (HEX-SSCP) detection and semi-automated DNA sequencing techniques. These procedures were applied to identify any known and/or novel variations in the genes that may be associated with a predisposition to hereditary haemochromatosis in non-Caucasian individuals. Various mobility shifts were identified by HEX-SSCP analysis. Variants were confirmed in the *HJV*, *HAMP*, *HMOX1*, *SLC40A1*, and *HFE* promoter regions using bi-directional semi-automated DNA sequencing. Variants were also identified in the coding regions of the *HMOX1*, *SLC40A1*, *CYBRD1* and *HJV* genes. The extended family of

the probands will be screened for the various variants found to define haplotypes that may contribute to the pathogenesis of haemochromatosis in this family. Our findings could contribute to elucidating the cause of non-*HFE* related iron-overload in non-Caucasian populations.